EXHIBIT A

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(54) **METHODS OF PRODUCING IMMUNOGLOBULINS, VECTORS AND TRANSFORMED HOST CELLS FOR USE THEREIN**

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(58) **Field of Search** 435/69.1, 69.7, 435/71.1, 70.1, 71.2, 320, 261, 252.1, 252.3, 81, 55, 56, 69.6, 252.33, 254.21, 483

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(57) **ABSTRACT**

The invention relates to processes for producing an immunoglobulin or an immunologically functional immunoglobulin fragment containing at least the variable domains of the immunoglobulin heavy and light chains. The processes can use one or more vectors which produce both the heavy and light chains or fragments thereof in a single cell. The invention also relates to the vectors used to produce the immunoglobulin or fragment, and to cells transformed with the vectors.

36 Claims, 19 Drawing Sheets

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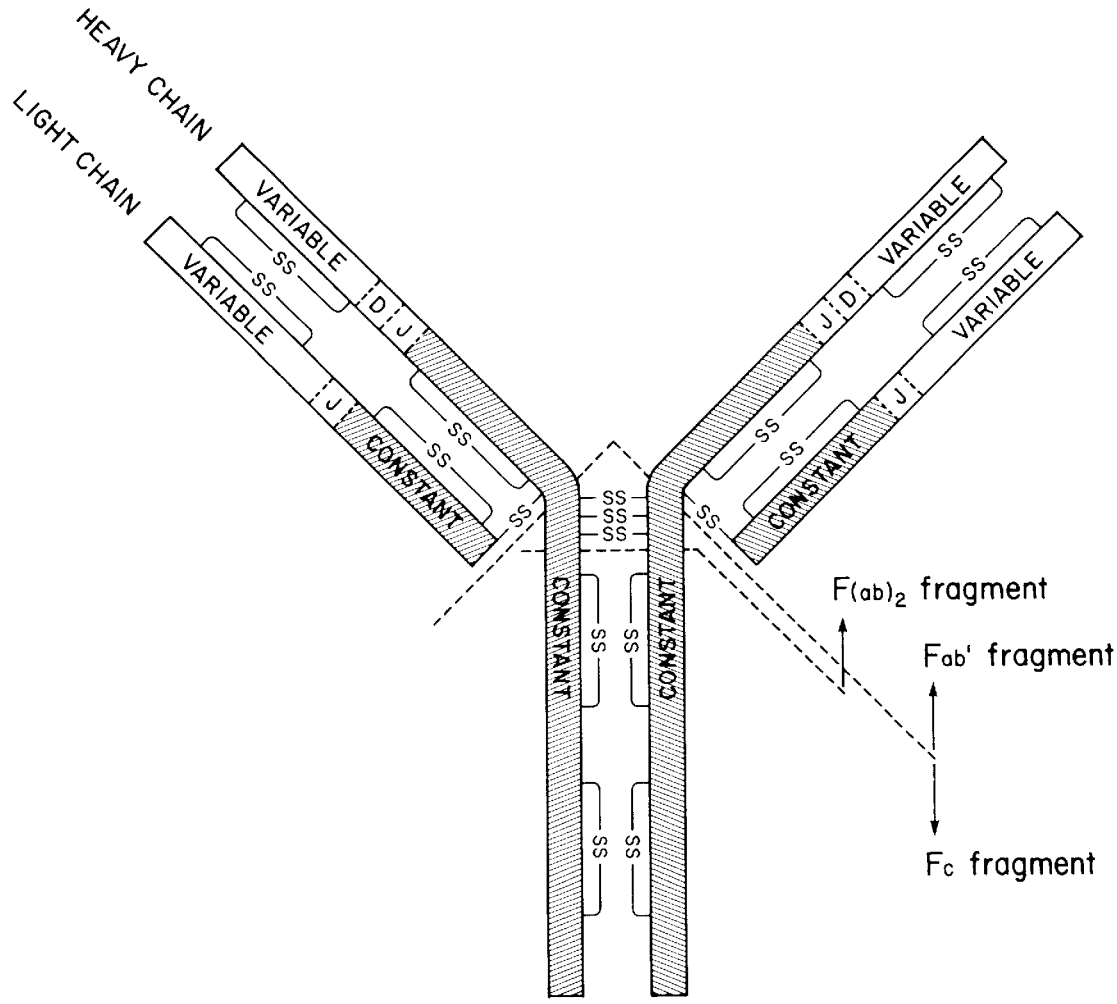
HEAVY CHAIN
LIGHT CHAIN
VARIABLE
CONSTANT
D
J
SS
F(ab)2 fragment
Fab' fragment
Fc fragment

Fig. 1.

```
                                                                                                              haeIII
                                               tth111                                tth111      hphI       haeI
1      GTTGCTGTGG TTGTCTGGTG TTGAAGGAGA CATTGTGATG ACCCAGTCTC ACAAATTCAT GTCCACATCA GTAGGAGACA GGGTCAGCAT CACCTGCAAG
       CAACGACACC AACAGACCAC AACTTCCTCT GTAACACTAC TGGGTCAGAG TGTTTAAGTA CAGGTGTAGT CATCCTCTGT CCCAGTCGTA GTGGACGTTC
                                                                                                    sfaNI

                                                                                                  scrFI
                          fnu4HI     scrFI                  scrFI                                 nciI
                 fokI     bbv        ecoRII                 ecoRII                                 hpaII      hinfI
101    GCCAGTCAGG ATGTGGGTGC TGCTATAGCC TGGTATCAAC AGAAACCAGG ACAATCTCCT AAACTACTGA TTTACTGGGC ATCCACCCGG CACACTGGAG
       CGGTCAGTCC TACACCCACG ACGATATCGG ACCATAGTTG TCTTTGGTCC TGTTAGAGGA TTTGATGACT AAATGACCCG TAGGTGGGCC GTGTGACCTC
                                                                                fokI      sfaNI

                                 xhoII
           sau3A                  sau3A
           dpnI                   dpnI                       hphI                                                hincII
201    TCCCTGATCG CTTCACAGGC AGTGGATCTG GGACAGATTT CACTCTCACC ATTAGCAATG TGCAGTCTGA TGACTTGGCA GATTATTTCT GTCAACAATA
       AGGGACTAGC GAAGTGTCCG TCACCTAGAC CCTGTCTAAA GTGAGAGTGG TAATCGTTAC ACGTCAGACT ACTGAACCGT CTAATAAAGA CAGTTGTTAT

                                     sau96                           fnu4HI                                         hpaI
                mnlI                 avaII   aluI    aluI  sfaNI      bbv                      mboII                hincII
301    TAGCGGGTAT CCTCTCACGT TCGGTGCTGG GACCAAGCTG GAGCTGAAAC GGGCTGATGC TGCACCAACT GTATCCATCT TCCCACCATC CAGTGAGCAG
       ATCGCCCATA GGAGAGTGCA AGCCACGACC CTGGTTCGAC CTCGACTTTG CCCGACTACG ACGTGGTTGA CATAGGTAGA AGGGTGGTAG GTCACTCGTC
                                                                                      fokI

                      mnlI
                mnlI   ddeI                    xmnI                                   mboII                       acyI
401    TTAACATCTG GAGGTGCCTC AGTCGTGTGC TTCTTGAACA ACTTCTACCC CAAAGACATC AATGTCAAGT GGAAGATTGA TGGCAGTGAA CGACAAAATG
       AATTGTAGAC CTCCACGGAG TCAGCACACG AAGAACTTGT TGAAGATGGG GTTTCTGTAG TTACAGTTCA CCTTCTAACT ACCGTCACTT GCTGTTTTAC
```

Fig. 2A.

```
                                  sau3A
                                  dpnI                                                  fnu4HI
         hgaI                   bclI                                                    bbv       mnlI   hincII                                alu I
501      GCGTCCTGAA CAGTTGGACT GATCAGGACA GCAAAGACAG CACCTACAGC ATGAGCAGCA CCCTCACGTT GACCAAGGAC GAGTATGAAC GACATAACAG
         CGCAGGACTT GTCAACCTGA CTAGTCCTGT CGTTTCTGTC GTGGATGTCG TACTCGTCGT GGGAGTGCAA CTGGTTCCTG CTCATACTTG CTGTATTGTC

                    mnlI                                                                                   sau96    hgaI
                      haeIII                                                                                   ddeI
                     haeI                        hphI             aluI                                     avaII    acyI
601      CTATACCTGT GAGGCCACTC ACAAGACATC AACTTCACCC ATTGTCAAGA GCTTCAACAG GAATGAGTGT TAGAGACAAA GGTCCTGAGA CGCCACCACC
         GATATGGACA CTCCGGTGAG TGTTCTGTAG TTGAAGTGGG TAACAGTTCT CGAAGTTGTC CTTACTCACA ATCTCTGTTT CCAGGACTCT GCGGTGGTGG

                                                                                                               mnlI
         aluI     aluI           mboII     ddeI           mnlI                                    hgiA      mnlI          mnlI
701      AGCTCCCCAG CTCCATCCTA TCTTCCCTTC TAAGGTCTTG GAGGCTTCCC CACAAGCGAC CTACCACTGT TGCGGTGCTC CAAACCTCCT CCCCACCTCC
         TCGAGGGGTC GAGGTAGGAT AGAAGGGAAG ATTCCAGAAC CTCCGAAGGG GTGTTCGCTG GATGGTGACA ACGCCACGAG GTTTGGAGGA GGGGTGGAGG
             fokI

            mnlI
         mnlI   mnlI                                       xmnI                   hinfI
801      TTCTCCTCCT CCTCCCTTTC CTTGGCTTTT ATCATGCTAA TATTTGCAGA AAATATTCAA TAAAGTGAGT CTTTGCACTT GA
         AAGAGGAGGA GGAGGGAAAG GAACCGAAAA TAGTACGATT ATAAACGTCT TTTATAAGTT ATTTCACTCA GAAACGTGAA CT

nucleotides: 882
```

Fig. 2B.

```
     -9                                1                                  10                                  20
    leu leu trp leu ser gly val glu gly asp ile val met thr gln ser his lys phe met ser thr ser val gly asp arg val ser
G   UUG CUG UGG UUG UCU GGU GUU GAA GGA GAC AUU GUG AUG ACC CAG UCU CAC AAA UUC AUG UCC ACA UCA GUA GGA GAC AGG GUC AGC

                                    30                                  40                                      50
ile thr cys lys ala ser gln asp val gly ala ala ile ala trp tyr gln gln lys pro gly gln ser pro lys leu leu ile tyr trp
AUC ACC UGC AAG GCC AGU CAG GAU GUG GGU GCU GCU AUA GCC UGG UAU CAA CAG AAA CCA GGA CAA UCU CCU AAA CUA CUG AUU UAC UGG

                                    60                                  70                                      80
ala ser thr arg his thr gly val pro asp arg phe thr gly ser gly ser gly thr asp phe thr leu thr ile ser asn val gln ser
GCA UCC ACC CGG CAC ACU GGA GUC CCU GAU CGC UUC ACA GGC AGU GGA UCU GGG ACA GAU UUC ACU CUC ACC AUU AGC AAU GUG CAG UCU

                                    90                                  100                                     110
asp asp leu ala asp tyr phe cys gln gln tyr ser gly tyr pro leu thr phe gly ala gly thr lys leu glu leu lys arg ala asp
GAU GAC UUG GCA GAU UAU UUC UGU CAA CAA UAU AGC GGG UAU CCU CUC ACG UUC GGU GCU GGG ACC AAG CUG GAG CUG AAA CGG GCU GAU

                                    120                                 130                                     140
ala ala pro thr val ser ile phe pro pro ser ser glu gln leu thr ser gly gly ala ser val val cys phe leu asn asn phe tyr
GCU GCA CCA ACU GUA UCC AUC UUC CCA CCA UCC AGU GAG CAG UUA ACA UCU GGA GGU GCC UCA GUC GUG UGC UUC UUG AAC AAC UUC UAC

                                    150                                 160                                     170
pro lys asp ile asn val lys trp lys ile asp gly ser glu arg gln asn gly val leu asn ser trp thr asp gln asp ser lys asp
CCC AAA GAC AUC AAU GUC AAG UGG AAG AUU GAU GGC AGU GAA CGA CAA AAU GGC GUC CUG AAC AGU UGG ACU GAU CAG GAC AGC AAA GAC

                                    180                                 190                                     200
ser thr tyr ser met ser ser thr leu thr leu thr lys asp glu tyr glu arg his asn ser tyr thr cys glu ala thr his lys thr
AGC ACC UAC AGC AUG AGC AGC ACC CUC ACG UUG ACC AAG GAC GAG UAU GAA CGA CAU AAC AGC UAU ACC UGU GAG GCC ACU CAC AAG ACA

                                    210             214
ser thr ser pro ile val lys ser phe asn arg asn glu cys AM
UCA ACU UCA CCC AUU GUC AAG AGC UUC AAC AGG AAU GAG UGU UAG AGACAAAGGUCCUGAGACGCCACCACCAGCUCCCCAGCUCCAUCCUAUCUUCCCUUCUAA

GGUCUUGGAGGCUUCCCCACAAGCGACCUACCACUGUUGCGGUGCUCCAAACCUCCUCCCCACCUCCUUCUCCUCCUCCUCCCUUUCCUUGGCUUUUAUCAUGCUAAUAUUUGCAGAAAA

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```
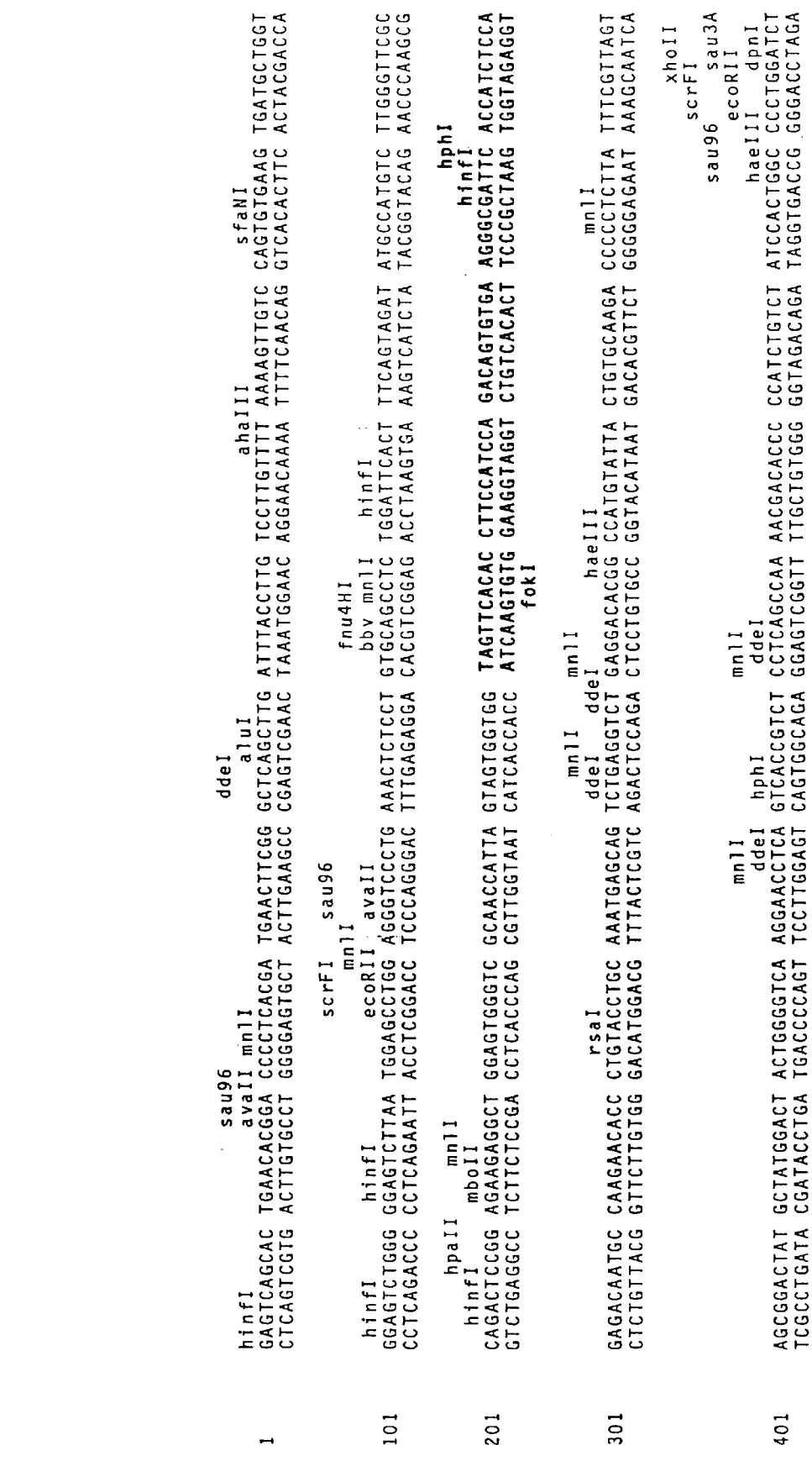

Fig. 3


1
101
201
301
401
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sau96
avaII mnlI
aluI
ddeI
ahaIII
sfaNI
scrFI sau96
mnlI
ecoRII avaII
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hinfI
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hpaII mnlI
hinfI mboII
hphI
hinfI
mnlI
ddeI
fokI
mnlI
ddeI ddeI
haeIII
mnlI
rsaI
mnlI
ddeI
mnlI
ddeI
hphI
mnlI
ddeI
haeIII
scrFI
sau96 sau3A
ecoRII
dpnI
xhoII

Fig. 4A.

```
                                     scrFI                                                               xhoII
                          ncoI   sfaNI    fokI                                                            sau3A
         fnu4HI                hphI  ecoRII    scrFI                                           scrFI      dpnI
         bbv                   bstEII          ecoRII                ddeI                      ecoRII    bamHI
501      GCTGCCCAAA CTAACTCCAT GGTGACCCTG GGATGCCTGG TCAAGGGCTA TTTCCCTGAG CCAGTGACAG TGACCTGGAA CTCTGGATCC CTGTCCAGCG
         CGACGGGTTT GATTGAGGTA CCACTGGGAC CCTACGGACC AGTTCCCGAT AAAGGGACTC GGTCACTGTC ACTGGACCTT GAGACCTAGG GACAGGTCGC

                                                             fnu4HI                            sau96
                         pvuII                               bbv  ddeI                     mnlI
           hgiA           aluI    pstI        mnlI        ddeI   aluI            mnlI          haeIII          hphI
601      GTGTGCACAC CTTCCCAGCT GTCCTGCAGT CTGACCTCTA CACTCTGAGC AGCTCAGTGA CTGTCCCCTC CAGCCCTCGG CCCAGCGAGA CCGTCACCTG
         CACACGTGTG GAAGGGTCGA CAGGACGTCA GACTGGAGAT GTGAGACTCG TCGAGTCACT GACAGGGGAG GTCGGGAGCC GGGTCGCTCT GGCAGTGGAC

                      scrFI
                         haeIII
                      nciI     fnu4HI                           scrFI
                bglI   hpaII   bbv                              ecoRII                      ndeI   rsaI
701      CAACGTTGCC CACCCGGCCA GCAGCACCAA GGTGGACAAG AAAATTGTGC CCAGGGATTG TGGTTGTAAG CCTTGCATAT GTACAGTCCC AGAAGTATCA
         GTTGCAACGG GTGGGCCGGT CGTCGTGGTT CCACCTGTTC TTTTAACACG GGTCCCTAAC ACCAACATTC GGAACGTATA CATGTCAGGG TCTTCATAGT

                                                                    mstII                                  sau3A
                                                 hphI           hinfI                                      dpnI  mnlI
             mboII  mboII                fokI hgiA                   ddeI                   accI         fokI   avaI
801      TCTGTCTTCA TCTTCCCCCC AAAGCCCAAG GATGTGCTCA CCATTACTCT GACTCCTAAG GTCACGTGTG TTGTGGTAGA CATCAGCAAG GATGATCCCG
         AGACAGAAGT AGAAGGGGGG TTTCGGGTTC CTACACGAGT GGTAATGAGA CTGAGGATTC CAGTGCACAC AACACCATCT GTAGTCGTTC CTACTAGGGC

                                                                       smaI
                                                                        scrFI
                                                                       scrFI
                                                                        nciI
                                                                       nciI
          sau96      pvuII                     mnlI          ddeI       hpaII
          avaII       aluI                        hgiA      aluI  hgaI     avaI  mnlI                     ddeI
901      AGGTCCAGTT CAGCTGGTTT GTAGATGATG TGGAGGTGCA CACAGCTCAG ACGCAACCCC GGGAGGAGCA GTTCAACAGC ACTTTCCGCT CAGTCAGTGA
         TCCAGGTCAA GTCGACCAAA CATCTACTAC ACCTCCACGT GTGTCGAGTC TGCGTTGGGG CCCTCCTCGT CAAGTTGTCG TGAAAGGCGA GTCAGTCACT
```

Fig. 4B.

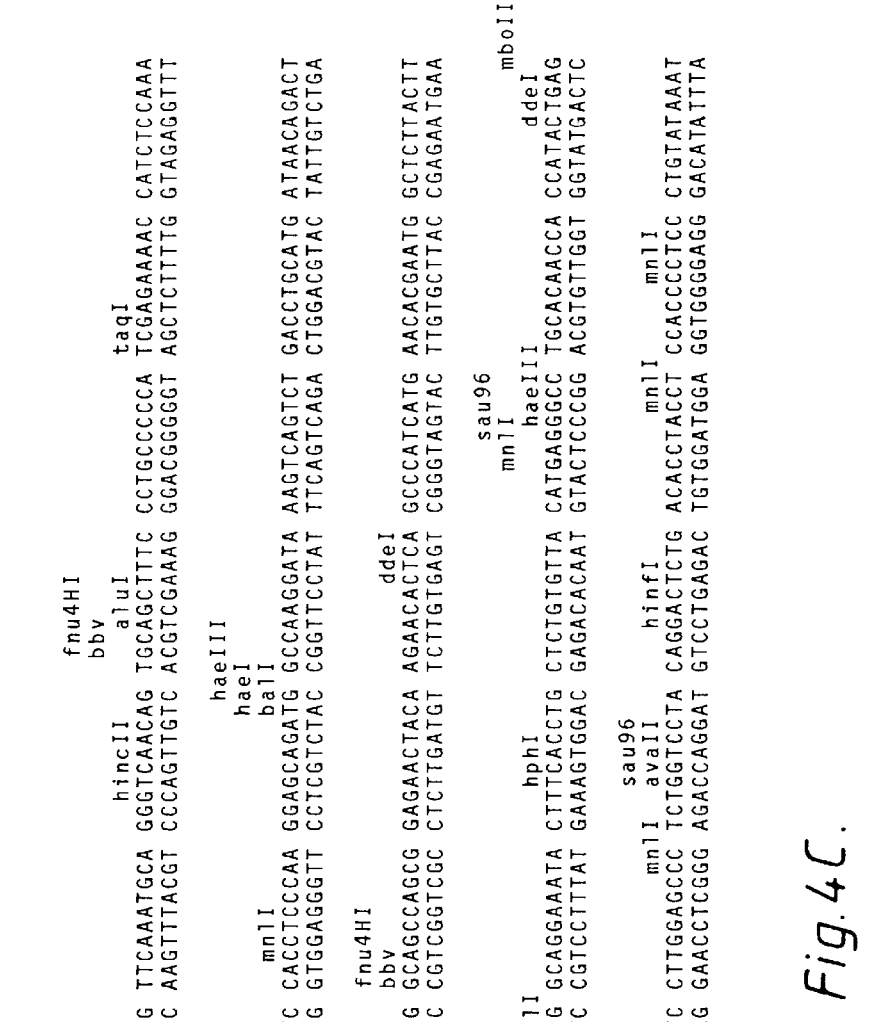

Fig. 4C.

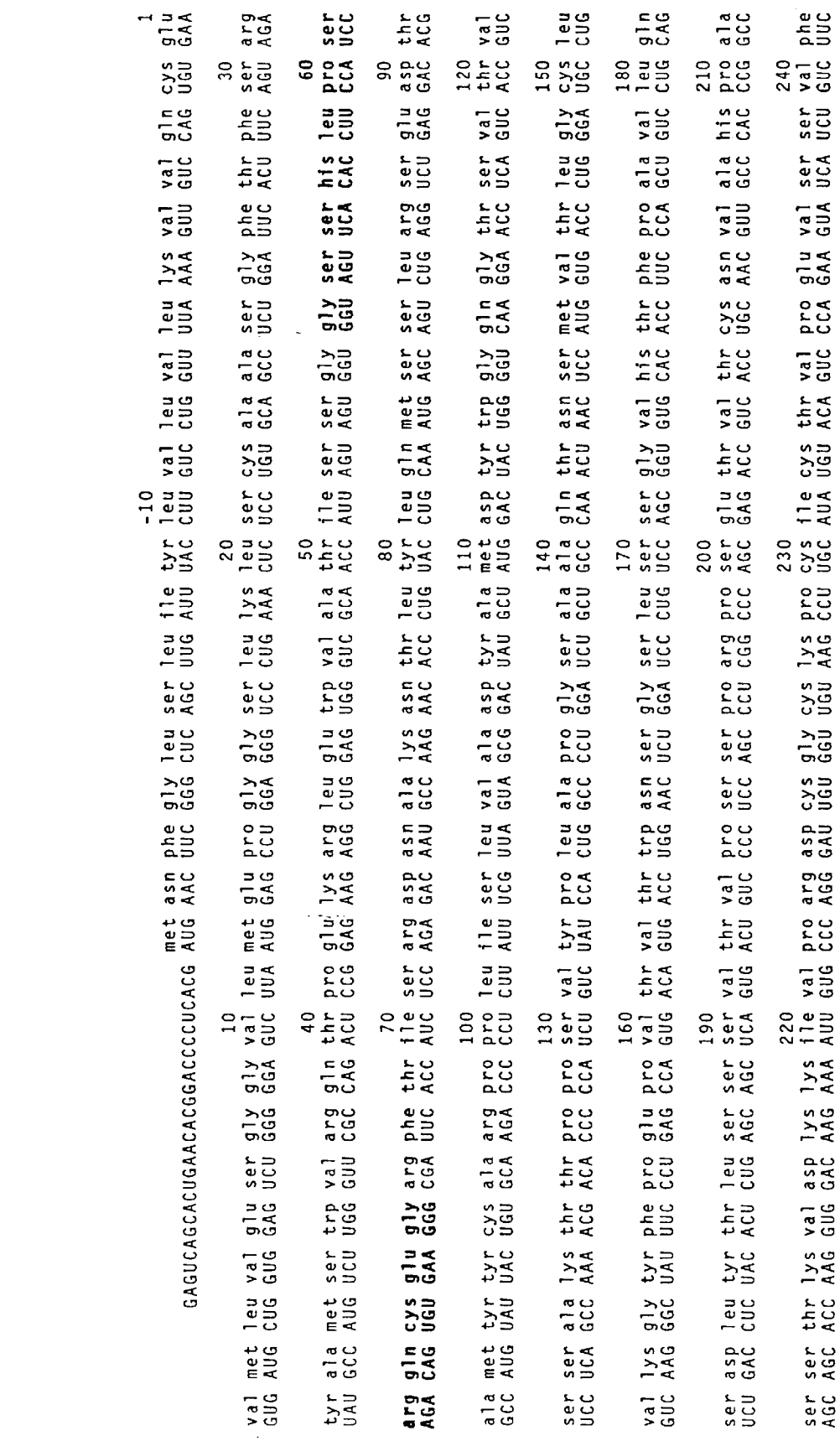
-10 1
met asn phe gly leu ser leu ile tyr leu val leu val leu lys val val gln cys glu
GAGUCAGCACUGAACACGGACCCCUCACG AUG AAC UUC GGG CUC AGC UUG AUU UAC CUU GUC CUG GUU UUA AAA GUU GUC CAG UGU GAA
10 20 30
val met leu val glu ser gly gly val leu met glu pro gly gly ser leu lys leu ser cys ala ala ser gly phe thr phe ser arg
GUG AUG CUG GUG GAG UCU GGG GGA GUC UUA AUG GAG CCU GGA GGG UCC CUG AAA CUC UCC UGU GCA GCC UCU GGA UUC ACU UUC AGU AGA
40 50 60
tyr ala met ser trp val arg gln thr pro glu lys arg leu glu trp val ala thr ile ser ser gly gly ser ser his leu pro ser
UAU GCC AUG UCU UGG GUU CGC CAG ACU CCG GAG AAG AGG CUG GAG UGG GUC GCA ACC AUU AGU AGU GGU AGU UCA CAC CUU CCA UCC
70 80 90
arg gln cys glu gly arg phe thr ile ser arg asp asn ala lys asn thr leu tyr leu gln met ser ser leu arg ser glu asp thr
AGA CAG UGU GAA GGG CGA UUC ACC AUC UCC AGA GAC AAU GCC AAG AAC ACC CUG UAC CUG CAA AUG AGC AGU CUG AGG UCU GAG GAC ACG
100 110 120
ala met tyr tyr cys ala arg pro pro leu ile ser leu val ala asp tyr ala met asp tyr trp gly gln gly thr ser val thr val
GCC AUG UAU UAC UGU GCA AGA CCC CCU CUU AUU UCG UUA GUA GCG GAC UAU GCU AUG GAC UAC UGG GGU CAA GGA ACC UCA GUC ACC GUC
130 140 150
ser ser ala lys thr thr pro pro ser val tyr pro leu ala pro gly ser ala ala gln thr asn ser met val thr leu gly cys leu
UCC UCA GCC AAA ACG ACA CCC CCA UCU GUC UAU CCA CUG GCC CCU GGA UCU GCU GCC CAA ACU AAC UCC AUG GUG ACC CUG GGA UGC CUG
160 170 180
val lys gly tyr phe pro glu pro val thr val thr trp asn ser gly ser leu ser ser gly val his thr phe pro ala val leu gln
GUC AAG GGC UAU UUC CCU GAG CCA GUG ACA GUG ACC UGG AAC UCU GGA UCC CUG UCC AGC GGU GUG CAC ACC UUC CCA GCU GUC CUG CAG
190 200 210
ser asp leu tyr thr leu ser ser ser val thr val pro ser ser pro arg pro ser glu thr val thr cys asn val ala his pro ala
UCU GAC CUC UAC ACU CUG AGC AGC UCA GUG ACU GUC CCC UCC AGC CCU CGG CCC AGC GAG ACC GUC ACC UGC AAC GUU GCC CAC CCG GCC
220 230 240
ser ser thr lys val asp lys lys ile val pro arg asp cys gly cys lys pro cys ile cys thr val pro glu val ser ser val phe
AGC AGC ACC AAG GUG GAC AAG AAA AUU GUG CCC AGG GAU UGU GGU UGU AAG CCU UGC AUA UGU ACA GUC CCA GAA GUA UCA UCU GUC UUC

Fig. 5A.

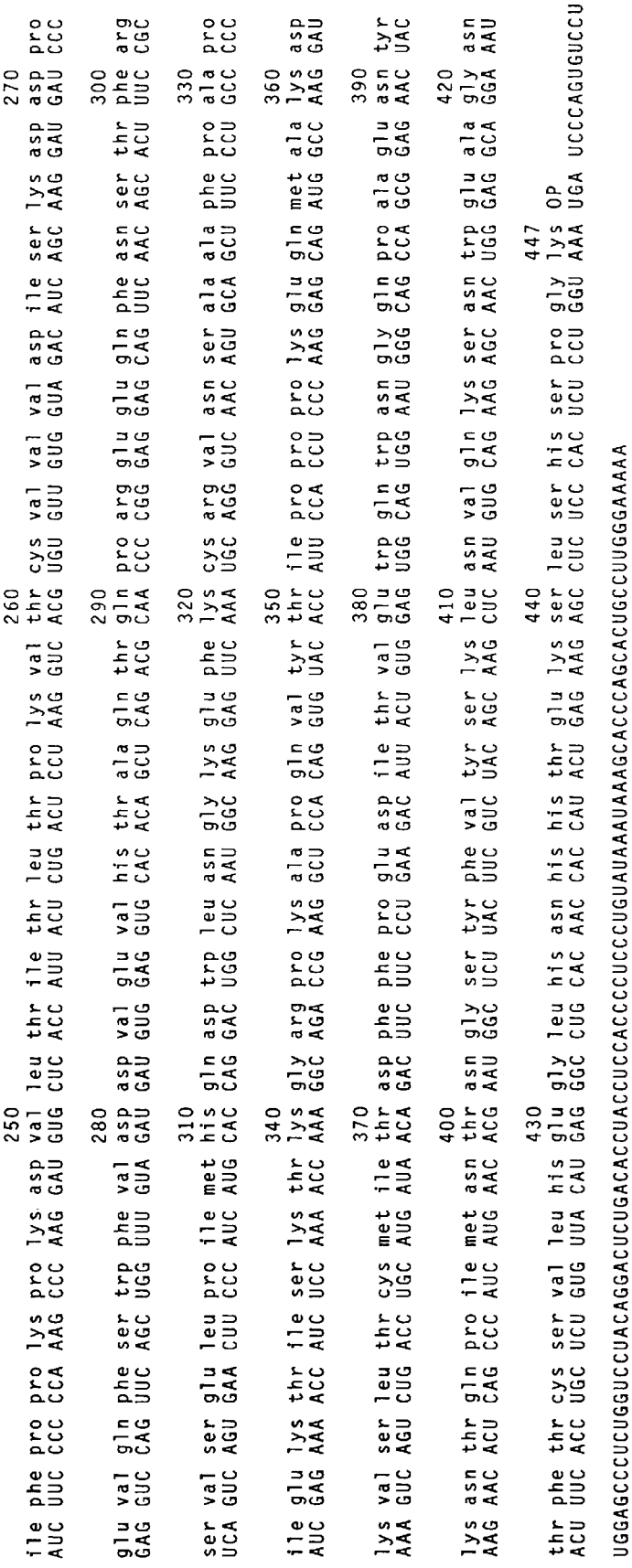
250 260 270
ile phe pro pro lys pro lys asp val leu thr ile thr leu thr pro lys val thr cys val val asp ile ser lys asp asp pro
AUC UUC CCC CCA AAG CCC AAG GAU GUG CUC ACC AUU ACU CUG ACU CCU AAG GUC ACG UGU GUU GUG GUA GAC AUC AGC AAG GAU GAU CCC
280 290 300
glu val gln phe ser trp phe val asp asp val glu val his thr ala gln thr gln pro arg glu glu gln phe asn ser thr phe arg
GAG GUC CAG UUC AGC UGG UUU GUA GAU GAU GUG GAG GUG CAC ACA GCU CAG ACG CAA CCC CGG GAG GAG CAG UUC AAC AGC ACU UUC CGC
310 320 330
ser val ser glu leu pro ile met his gln asp trp leu asn gly lys glu phe lys cys arg val asn ser ala ala phe pro ala pro
UCA GUC AGU GAA CUU CCC AUC AUG CAC CAG GAC UGG CUC AAU GGC AAG GAG UUC AAA UGC AGG GUC AAC AGU GCA GCU UUC CCU GCC CCC
340 350 360
ile glu lys thr ile ser lys thr lys gly arg pro lys ala pro gln val tyr thr ile pro pro pro lys glu gln met ala lys asp
AUC GAG AAA ACC AUC UCC AAA ACC AAA GGC AGA CCG AAG GCU CCA CAG GUG UAC ACC AUU CCA CCU CCC AAG GAG CAG AUG GCC AAG GAU
370 380 390
lys val ser leu thr cys met ile thr asp phe phe pro glu asp ile thr val glu trp gln trp asn gly gln pro ala glu asn tyr
AAA GUC AGU CUG ACC UGC AUG AUA ACA GAC UUC UUC CCU GAA GAC AUU ACU GUG GAG UGG CAG UGG AAU GGG CAG CCA GCG GAG AAC UAC
400 410 420
lys asn thr gln pro ile met asn thr asn gly ser tyr phe val tyr ser lys leu asn val gln lys ser asn trp glu ala gly asn
AAG AAC ACU CAG CCC AUC AUG AAC ACG AAU GGC UCU UAC UUC GUC UAC AGC AAG CUC AAU GUG CAG AAG AGC AAC UGG GAG GCA GGA AAU
430 440 447
thr phe thr cys ser val leu his glu gly leu his asn his thr glu lys ser leu ser his ser pro gly lys OP
ACU UUC ACC UGC UCU GUG UUA CAU GAG GGC CUG CAC AAC CAC CAU ACU GAG AAG AGC CUC UCC CAC UCU CCU GGU AAA UGA UCCCAGUGUCCU
UGGAGCCCUCUGGUCCUACAGGACUCUGACACCUACCUCCACCCCUCCCUGUAUAAUAAAGCACCCAGCACUGCCUUGGGAAAAA

Fig. 5B.

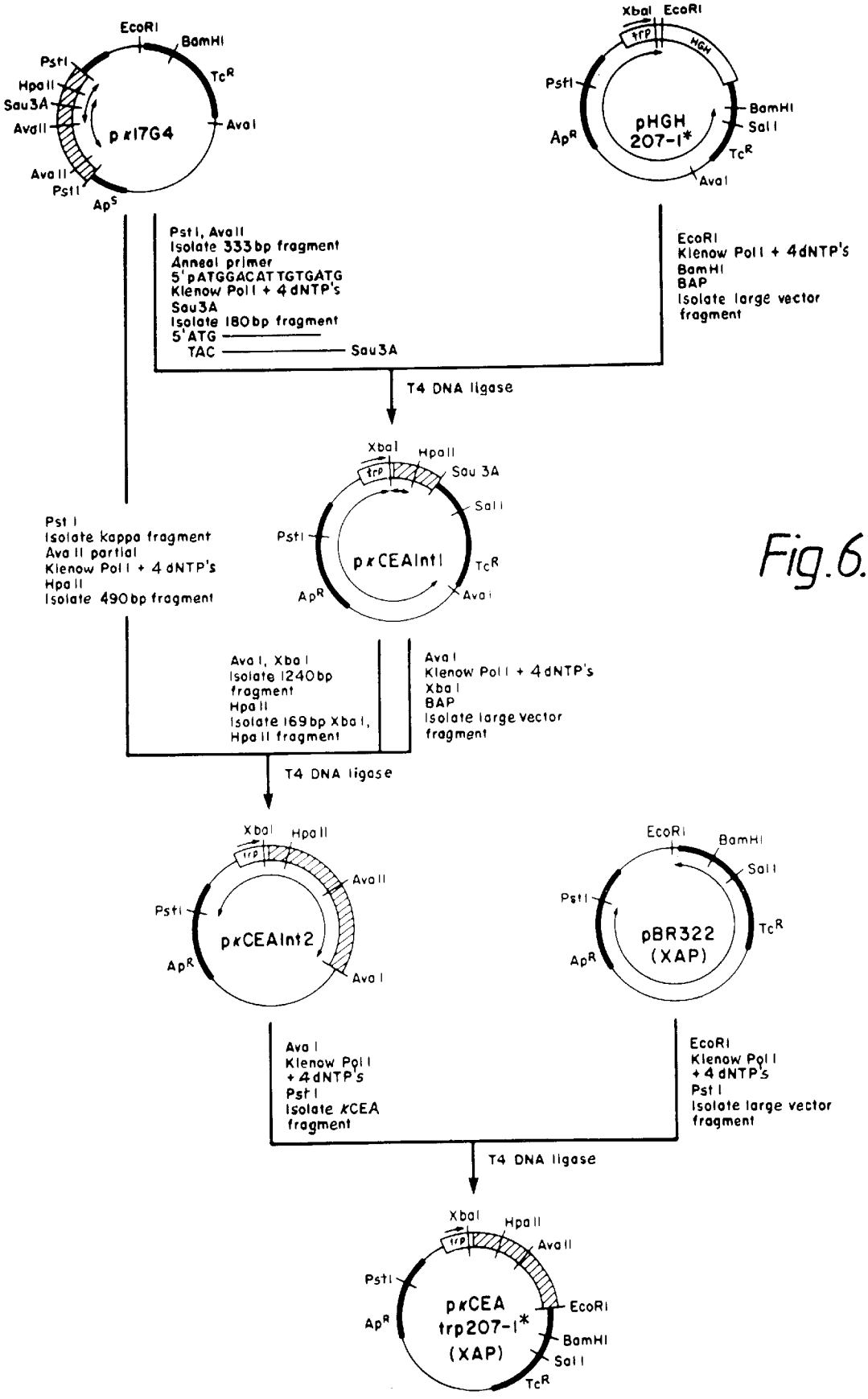
EcoRI
BamHI
PstI
HpaII
Sau3A
AvaII
TcR
AvaI
pκ17G4
ApS
AvaII
PstI
XbaI
EcoRI
trp
HGH
PstI
BamHI
SalI
ApR
pHGH
207-1*
TcR
AvaI
PstI, AvaII
Isolate 333bp fragment
Anneal primer
5'pATGGACATTGTGATG
Klenow PolI + 4dNTP's
Sau3A
Isolate 180bp fragment
5'ATG
TAC
Sau3A
EcoRI
Klenow PolI + 4dNTP's
BamHI
BAP
Isolate large vector
fragment
T4 DNA ligase
XbaI
HpaII
trp
Sau 3A
SalI
PstI
pκCEAInt1
TcR
ApR
AvaI
Pst I
Isolate kappa fragment
Ava II partial
Klenow PolI + 4 dNTP's
Hpa II
Isolate 490bp fragment
Ava I, Xba I
Isolate 1240bp
fragment
Hpa II
Isolate 169bp Xba I,
Hpa II fragment
Ava I
Klenow PolI + 4dNTP's
Xba I
BAP
Isolate large vector
fragment
T4 DNA ligase
XbaI
HpaII
trp
AvaII
PstI
pκCEAInt2
ApR
AvaI
EcoRI
BamHI
SalI
PstI
pBR322
(XAP)
TcR
ApR
Ava I
Klenow PolI
+4dNTP's
Pst I
Isolate κCEA
fragment
EcoRI
Klenow PolI
+4dNTP's
Pst I
Isolate large vector
fragment
T4 DNA ligase
XbaI
HpaII
trp
AvaII
PstI
pκCEA
trp207-1*
(XAP)
EcoRI
ApR
BamHI
SalI
TcR

Fig.6.

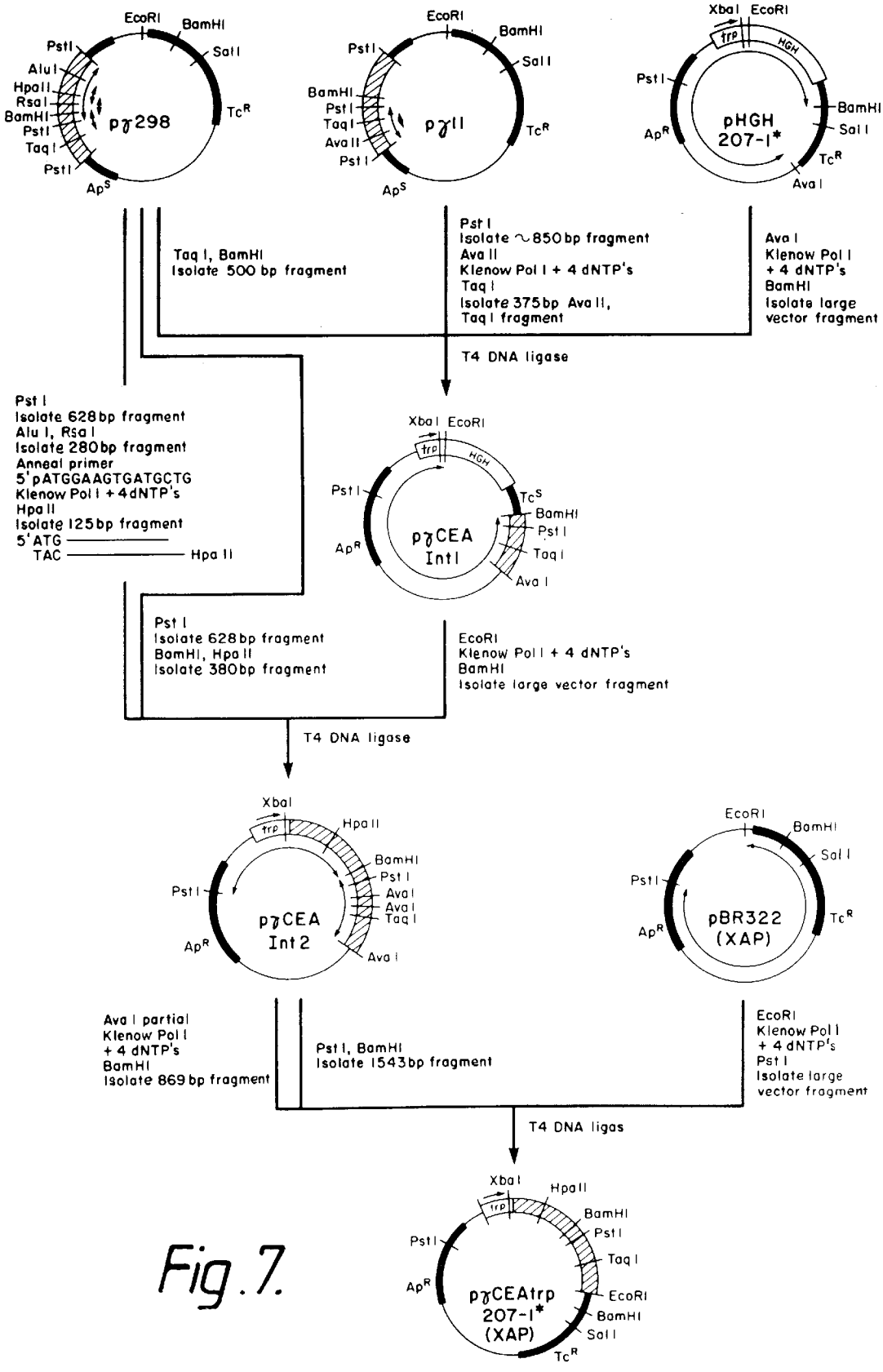
EcoRI
BamHI
Sal I
Tc^R
pγ298
Pst I
Alu I
Hpa II
Rsa I
BamHI
Pst I
Taq I
Pst I
Ap^S
pγII
Taq I, BamHI
Isolate 500 bp fragment
Pst I
Isolate ~850 bp fragment
Ava II
Klenow Pol I + 4 dNTP's
Taq I
Isolate 375 bp Ava II,
Taq I fragment
Xba I
EcoRI
trp
HGH
pHGH
207-1*
Ap^R
Ava I
Ava I
Klenow Pol I
+ 4 dNTP's
BamHI
Isolate large
vector fragment
T4 DNA ligase
Pst I
Isolate 628 bp fragment
Alu I, Rsa I
Isolate 280 bp fragment
Anneal primer
5'pATGGAAGTGATGCTG
Klenow Pol I + 4 dNTP's
Hpa II
Isolate 125 bp fragment
5'ATG
TAC
Hpa II
pγCEA
Int1
Tc^S
Taq I
Pst I
Isolate 628 bp fragment
BamHI, Hpa II
Isolate 380 bp fragment
EcoRI
Klenow Pol I + 4 dNTP's
BamHI
Isolate large vector fragment
T4 DNA ligase
Xba I
Hpa II
pγCEA
Int2
Ava I
Ava I partial
Klenow Pol I
+ 4 dNTP's
BamHI
Isolate 869 bp fragment
Pst I, BamHI
Isolate 1543 bp fragment
pBR322
(XAP)
EcoRI
Klenow Pol I
+ 4 dNTP's
Pst I
Isolate large
vector fragment
T4 DNA ligas
pγCEAtrp
207-1*
(XAP)

Fig. 7.

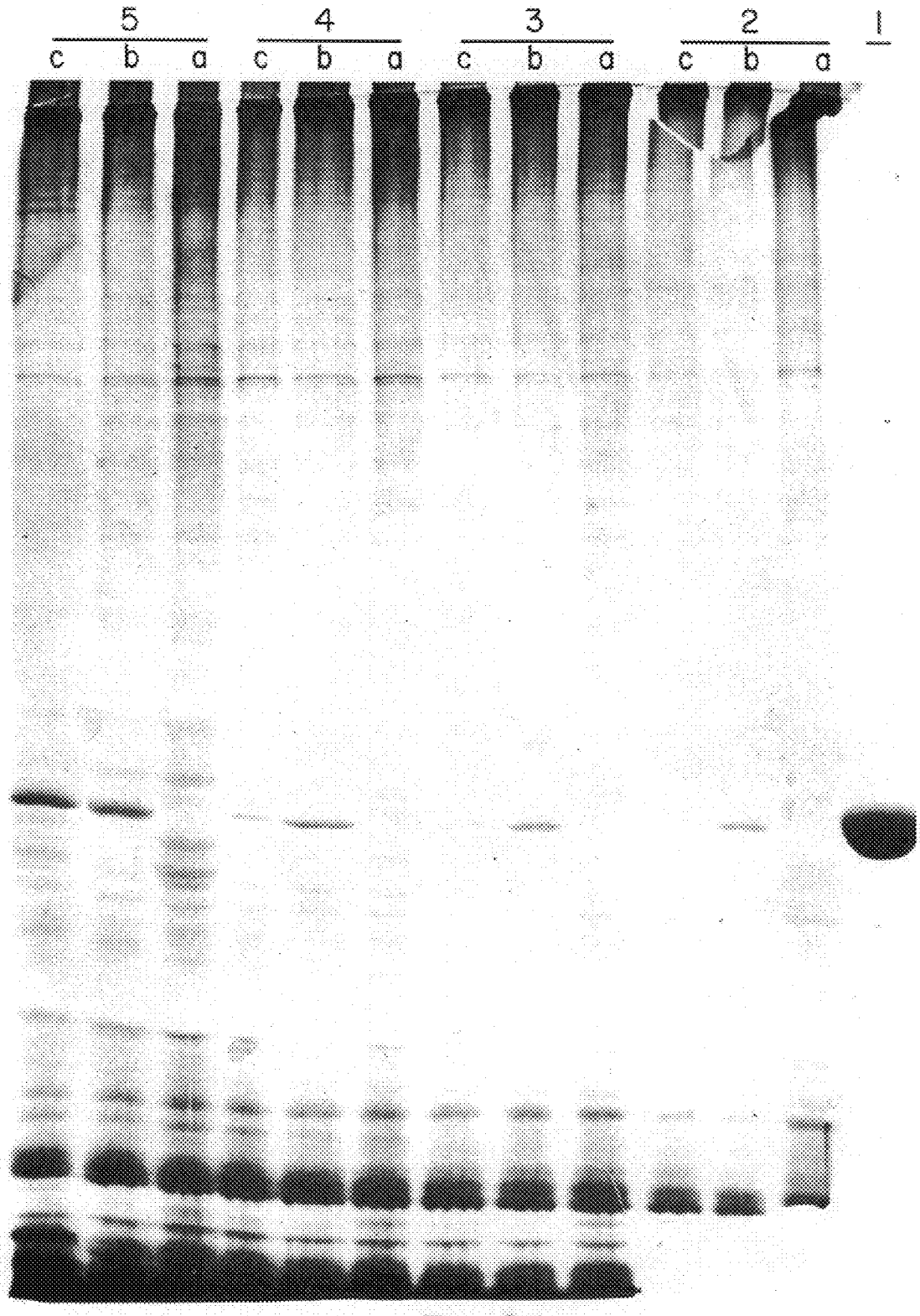

Fig. 8A.

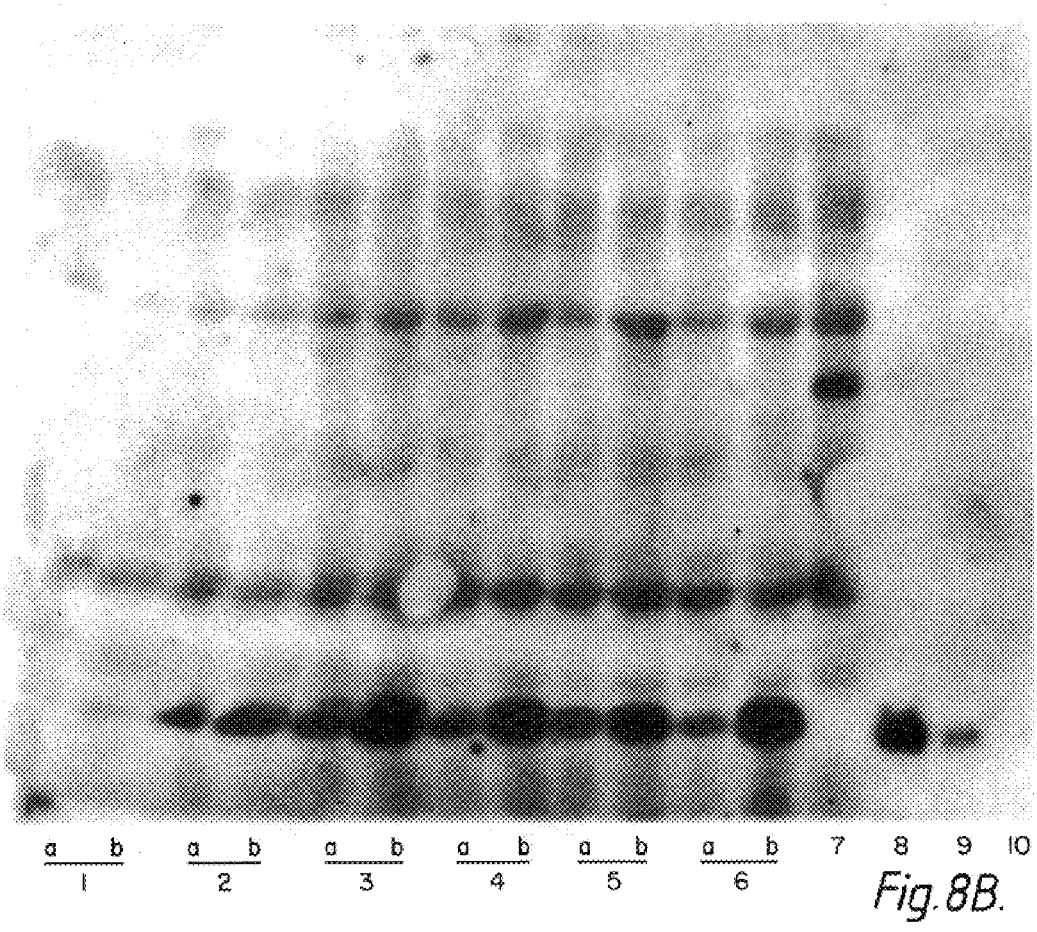

Fig. 8B.

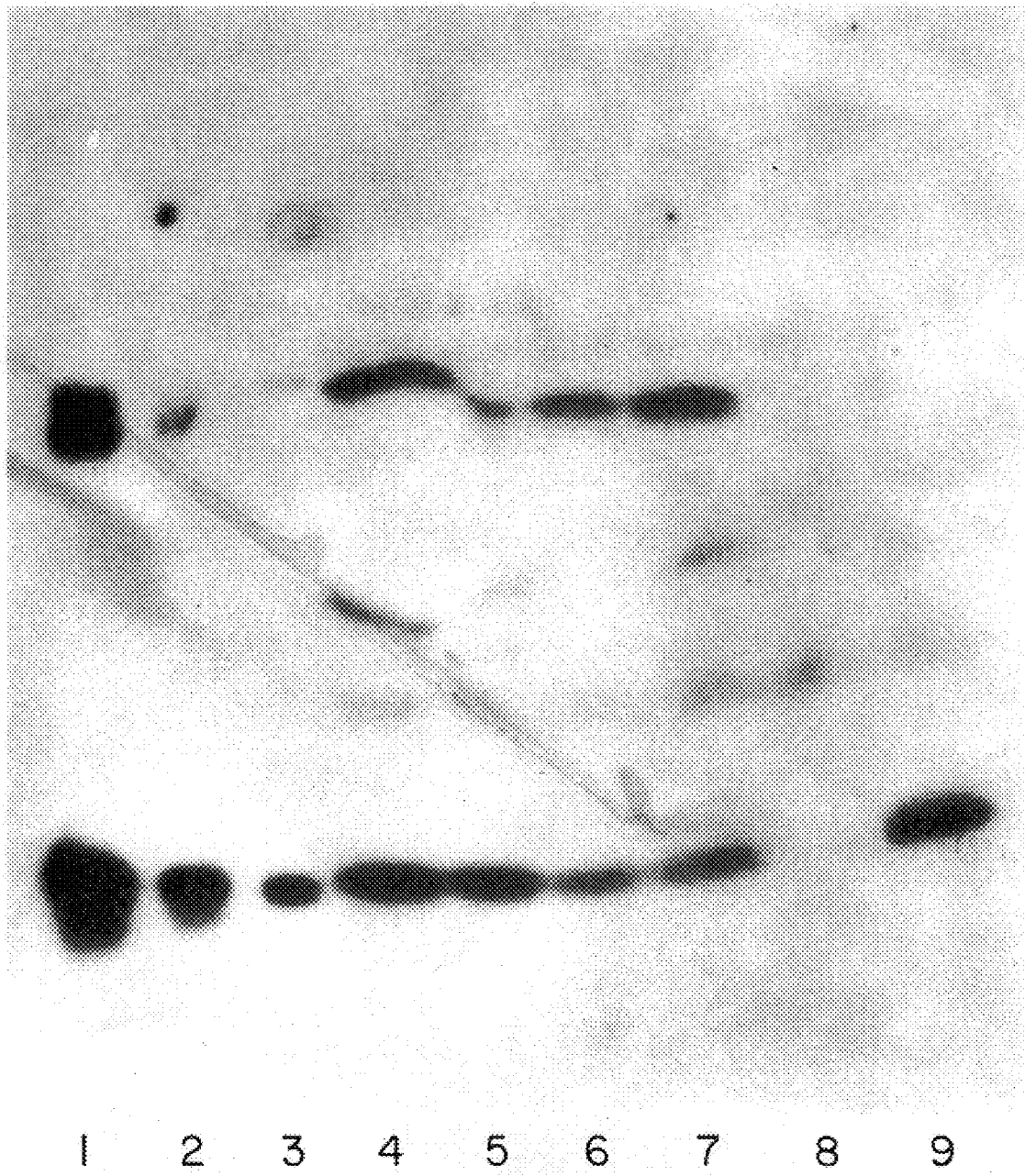

Fig. 8C.

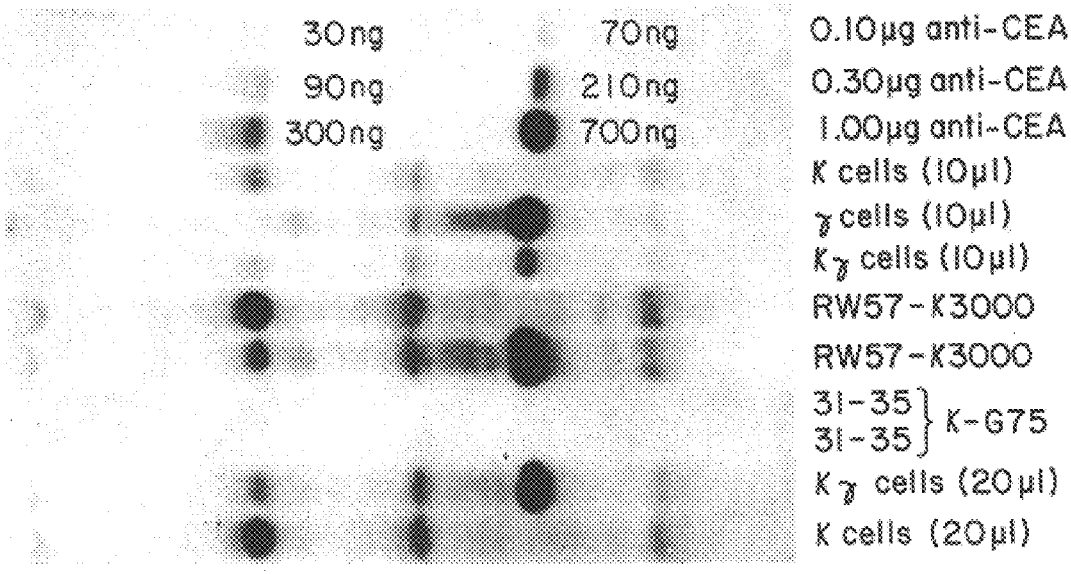
30ng
90ng
300ng
70ng
210ng
700ng
0.10µg anti-CEA
0.30µg anti-CEA
1.00µg anti-CEA
K cells (10µl)
γ cells (10µl)
Kγ cells (10µl)
RW57-K3000
RW57-K3000
31-35
31-35
K-675
Kγ cells (20µl)
K cells (20µl)

Fig. 9.

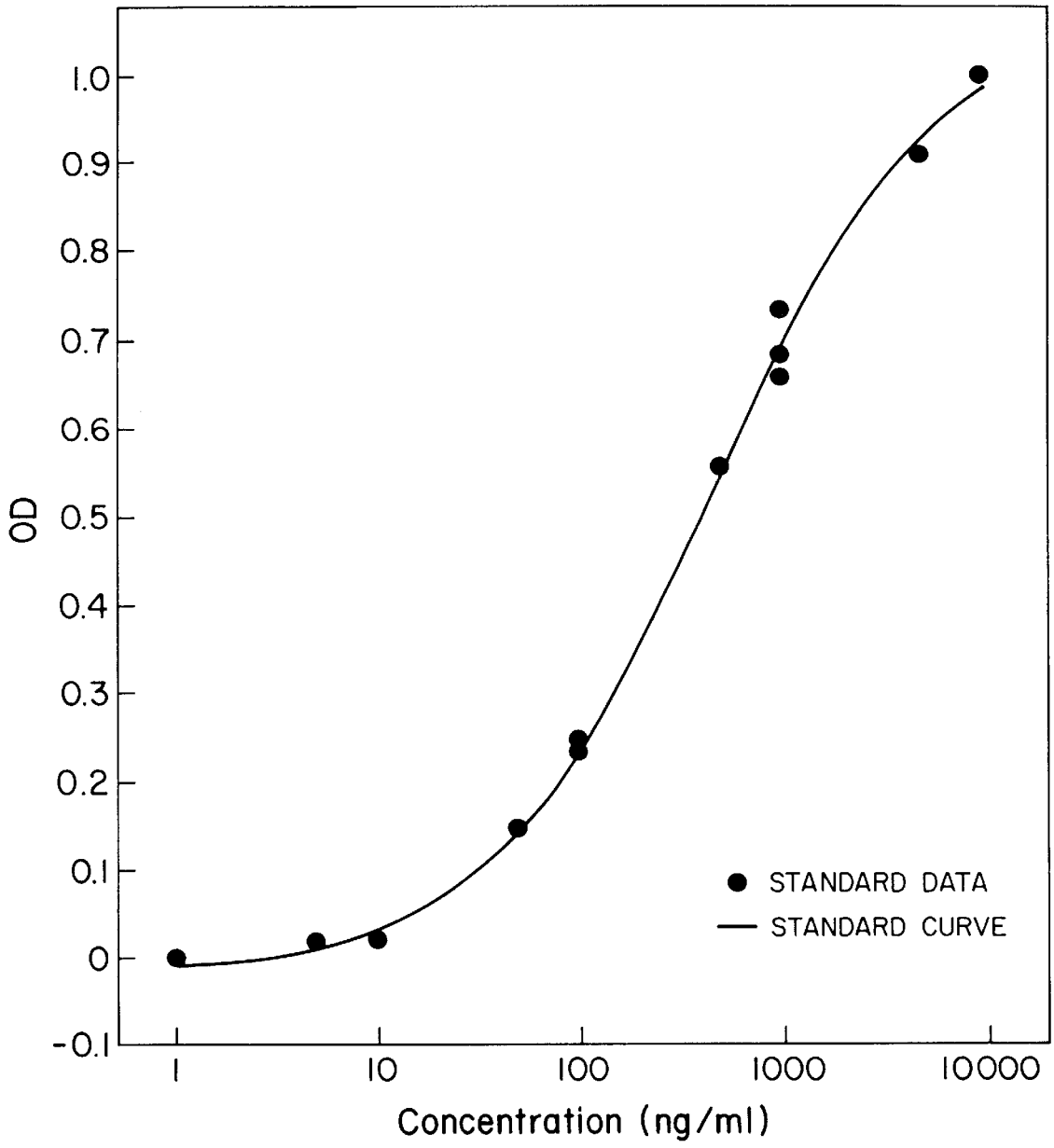
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
OD
1
10
100
1000
10000
Concentration (ng/ml)
STANDARD DATA
STANDARD CURVE

Fig. 10.

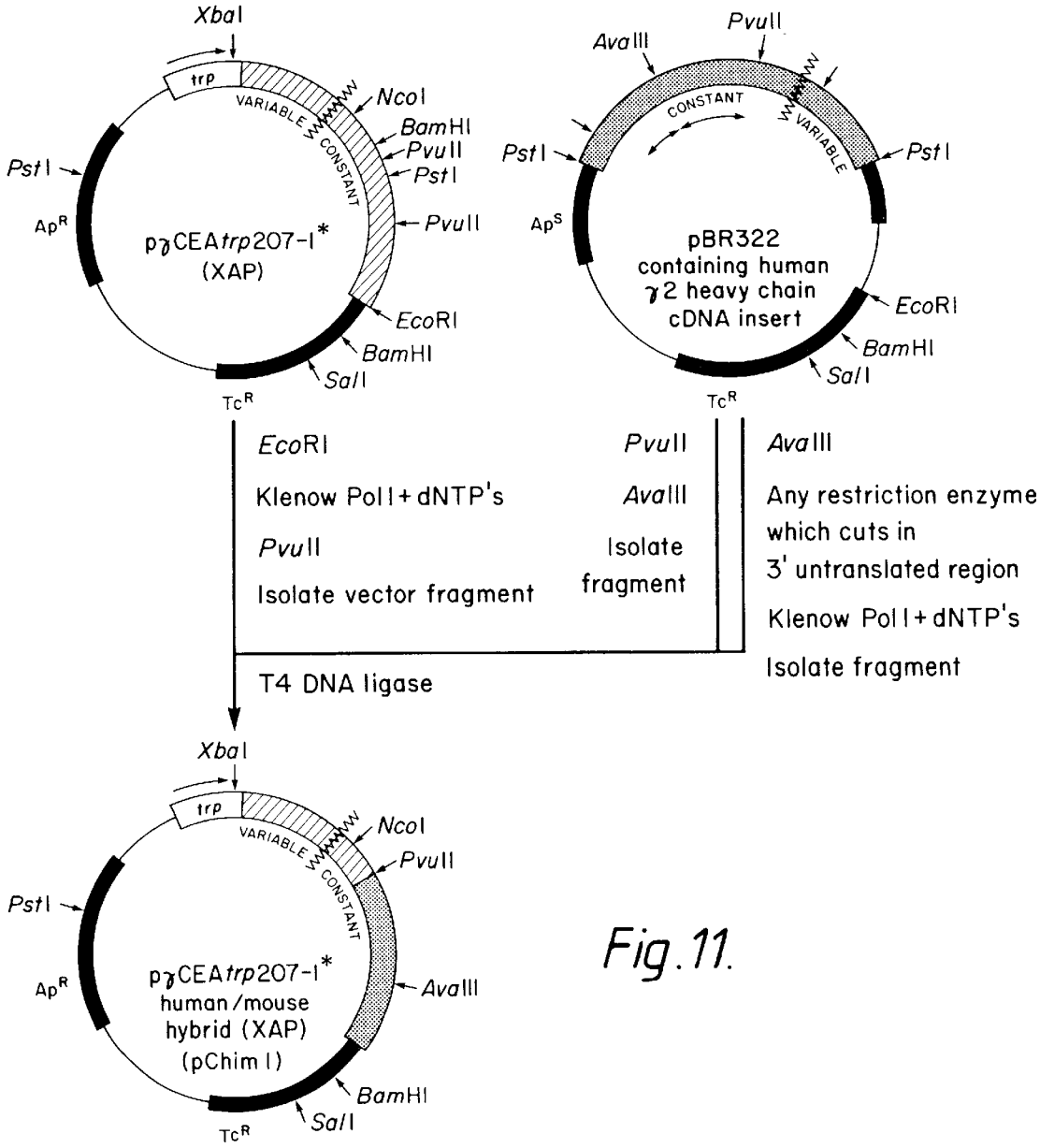
XbaI
trp
VARIABLE
CONSTANT
NcoI
BamHI
PvuII
PstI
PvuII
EcoRI
BamHI
SalI
TcR
PstI
ApR
pγCEAtrp207-1*
(XAP)
AvaIII
PvuII
CONSTANT
VARIABLE
PstI
PstI
ApS
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
TcR
EcoRI
Klenow PolI + dNTP's
PvuII
Isolate vector fragment
PvuII
AvaIII
Isolate
fragment
AvaIII
Any restriction enzyme
which cuts in
3' untranslated region
Klenow PolI + dNTP's
Isolate fragment
T4 DNA ligase
XbaI
trp
VARIABLE
CONSTANT
NcoI
PvuII
PstI
ApR
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim I)
AvaIII
BamHI
SalI
TcR

Fig. 11.

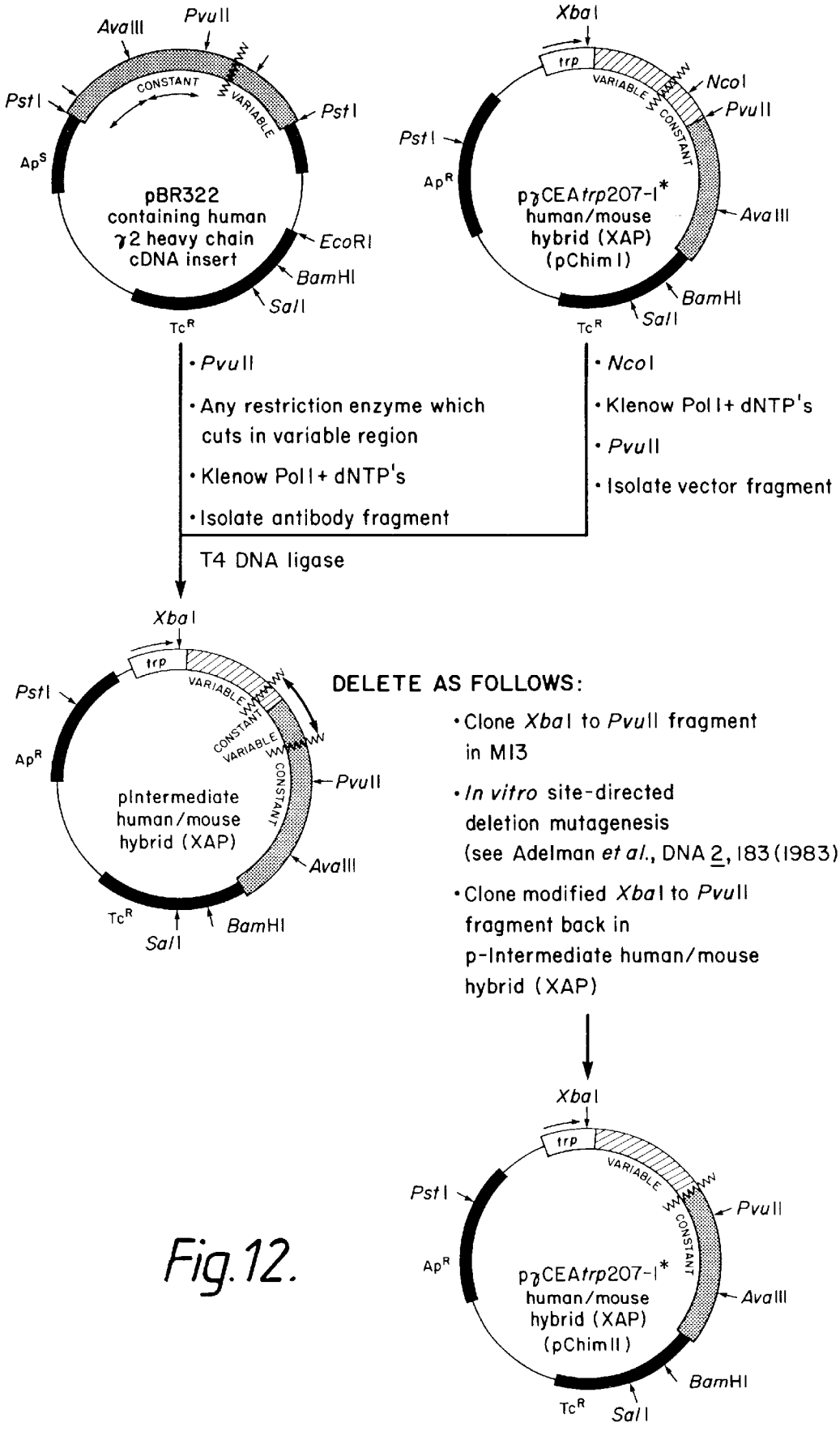
AvaIII
PvuII
PstI
CONSTANT
VARIABLE
PstI
ApS
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
TcR
XbaI
trp
VARIABLE
NcoI
PvuII
PstI
ApR
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim I)
AvaIII
BamHI
SalI
TcR
• PvuII
• Any restriction enzyme which cuts in variable region
• Klenow PolI + dNTP's
• Isolate antibody fragment
• NcoI
• Klenow PolI + dNTP's
• PvuII
• Isolate vector fragment
T4 DNA ligase
XbaI
trp
VARIABLE
CONSTANT
VARIABLE
CONSTANT
PstI
ApR
pIntermediate
human/mouse
hybrid (XAP)
PvuII
AvaIII
TcR
SalI
BamHI
DELETE AS FOLLOWS:
• Clone XbaI to PvuII fragment in M13
• In vitro site-directed deletion mutagenesis (see Adelman et al., DNA 2, 183 (1983))
• Clone modified XbaI to PvuII fragment back in p-Intermediate human/mouse hybrid (XAP)
XbaI
trp
VARIABLE
CONSTANT
PstI
ApR
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim II)
PvuII
AvaIII
BamHI
TcR
SalI

Fig. 12.

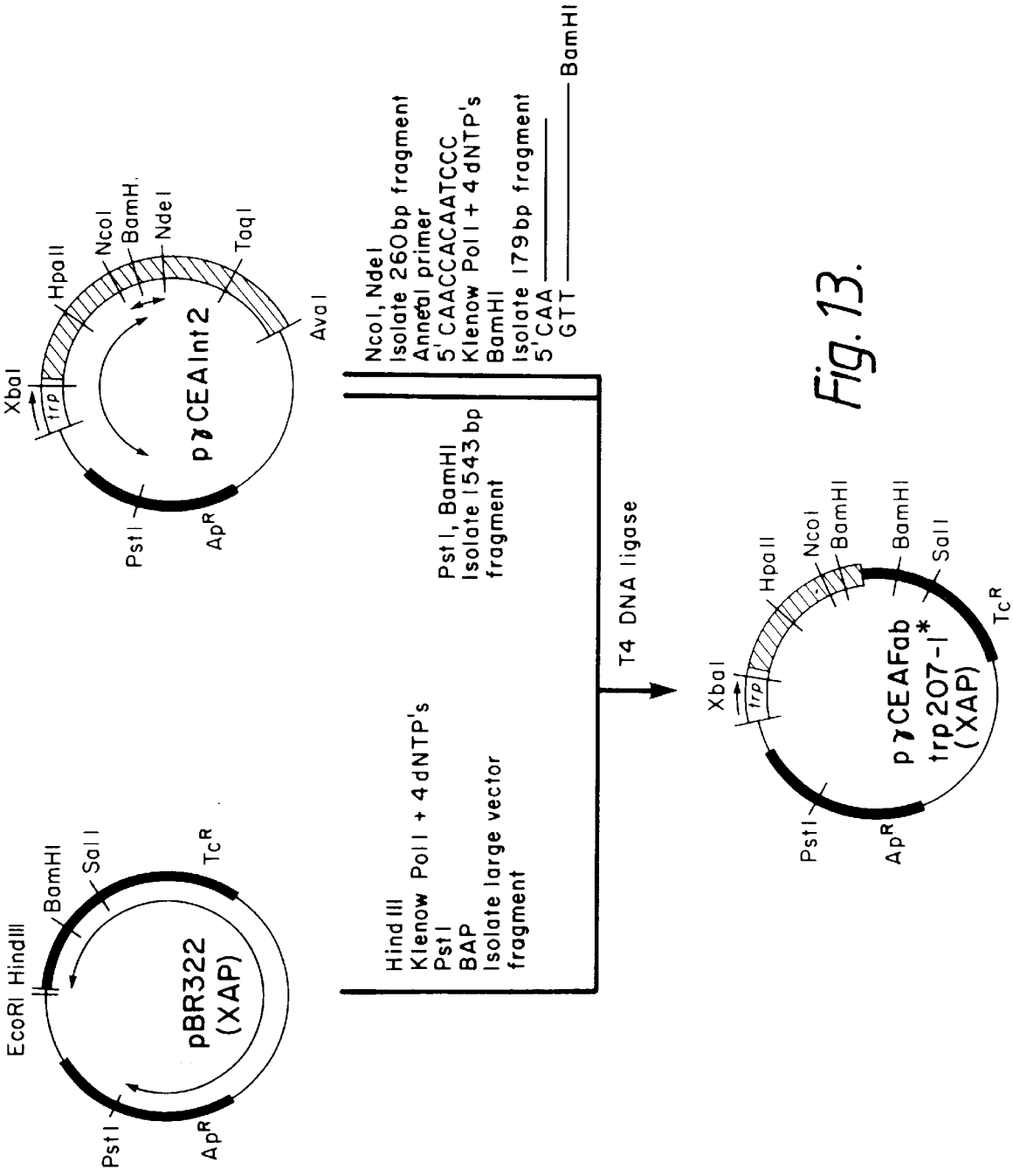
pγCEAInt2
XbaI
trp
HpaII
NcoI
BamH.
NdeI
TaqI
AvaI
PstI
ApR
NcoI, NdeI
Isolate 260bp fragment
Anneal primer
5' CAACCACAATCCC
Klenow PolI + 4 dNTP's
BamHI
Isolate 179 bp fragment
5' CAA
GTT
BamHI
PstI, BamHI
Isolate 1543 bp
fragment
HindIII
Klenow PolI + 4 dNTP's
PstI
BAP
Isolate large vector
fragment
T4 DNA ligase
pBR322
(XAP)
EcoRI HindIII
BamHI
SalI
TcR
PstI
ApR
pγCEAFab
trp207-1*
(XAP)
XbaI
trp
HpaII
NcoI
BamHI
BamHI
SalI
TcR
PstI
ApR

Fig. 13.

METHODS OF PRODUCING IMMUNOGLOBULINS, VECTORS AND TRANSFORMED HOST CELLS FOR USE THEREIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 06/483,457, filed Apr. 8, 1983, now U.S. Pat. No. 4,816,567, issued Mar. 28, 1989.

BACKGROUND OF THE INVENTION

This invention relates to the field of immunoglobulin production and to modification of naturally occurring immunoglobulin amino acid sequences. Specifically, the invention relates to using recombinant techniques to produce both immunoglobulins which are analogous to those normally found in vertebrate systems and to take advantage of these gene modification techniques to construct chimeric or other modified forms.

A. Immunoglobulins and Antibodies

Antibodies are specific immunoglobulin polypeptides produced by the vertebrate immune system in response to challenge by foreign proteins, glycoproteins, cells, or other antigenic foreign substances. The sequence of events which permits the organism to overcome invasion by foreign cells or to rid the system of foreign substances is at least partially understood. An important part of this process is the manufacture of antibodies which bind specifically to a particular foreign substance. The binding specificity of such polypeptides to a particular antigen is highly refined, and the multitude of specificities capable of being generated by the individual vertebrate is remarkable in its complexity and variability. Thousands of antigens are capable of eliciting responses, each almost exclusively directed to the particular antigen which elicited it.

Immunoglobulins include both antibodies, as above described, and analogous protein substances which lack antigen specificity. The latter are produced at low levels by the lymph system and in increased levels by myelomas.

A.1 Source and Utility

Two major sources of vertebrate antibodies are presently utilized—generation in situ by the mammalian B lymphocytes and in cell culture by B-cell hybrids. Antibodies are made in situ as a result of the differentiation of immature B lymphocytes into plasma cells, which occurs in response to stimulation by specific antigens. In the undifferentiated B cell, the portions of DNA coding for the various regions on the immunoglobulin chains are separated in the genomic DNA. The sequences are reassembled sequentially prior to transcription. A review of this process has been given by Gough, *Trends in Biochem Sci*, 6: 203 (1981). The resulting rearranged genome is capable of expression in the mature B lymphocyte to produce the desired antibody. Even when only a single antigen is introduced into the sphere of the immune system for a particular mammal, however, a uniform population of antibodies does not result. The in situ immune response to any particular antigen is defined by the mosaic of responses to the various determinants which are present on the antigen. Each subset of homologous antibody is contributed by a single population of B-cells—hence in situ generation of antibodies is "polyclonal".

This limited but inherent heterogeneity has been overcome in numerous particular cases by use of hybridoma technology to create "monoclonal" antibodies (Kohler, et al., *Eur. J. Immunol.*, 6: 511 (1976)). In this process, splenocytes

or lymphocytes from a mammal which has been injected with antigen are fused with a tumor cell line, thus producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell. The hybrids thus formed we segregated into single genetic strains by selection, dilution, and regrowth, and each strain thus represents a single genetic line. They therefore produce immunoreactive antibodies against a desired antigen which are assured to be homogenous, and which antibodies, referencing their pure genetic parentage, are called "monoclonal". Hybridoma technology has to this time been focused largely on the fusion of murine lines, but human-human hybridomas (Olsson, L. et al., *Proc. Natl. Acad. Sci.* (*USA*), 77: 5429 (1980)); human-murine hybridomas (Schlom, J. , et al. (ibid) 77: 6841 (1980)) and several other xenogenic hybrid combinations have been prepared as well. Alternatively, primary, antibody producing, B cells have been immortalized in vitro by transformation with viral DNA.

Polyclonal, or, much more preferably monoclonal, antibodies have a variety of useful properties similar to those of the present invention. For example, they can be used as specific immunoprecipitating reagents to detect the presence of the antigen which elicited the initial processing of the B cell genome by coupling this antigen-antibody reaction with suitable detection techniques such as labeling with radioisotopes or with enzymes capable of assay (RIA, EMIT, and ELISA). Antibodies are thus the foundation of immuno diagnostic tests for many antigenic substances. In another important use, antibodies can be directly injected into subjects suffering from an attack by a substance or organism containing the antigen in question to combat this attack. This process is currently in its experimental stages, but its potential is clearly seen. Third, whole body diagnosis and treatment is made possible because injected antibodies are directed to specific target disease tissues, and thus can be used either to determine the presence of the disease by carrying with them a suitable label, or to attack the diseased tissue by carrying a suitable drug.

Monoclonal antibodies produced by hybridomas, while theoretically effective as suggested above and clearly preferable to polyclonal antibodies because of their specificity, suffer from certain disadvantages. First, they tend to be contaminated with other proteins and cellular materials of hybridoma, (and, therefore, mammalian) origin. These cells contain additional materials, notably nucleic acid fragments, but protein fragments as well, which are capable of enhancing, causing, or mediating carcinogic responses. Second, hybridoma lines producing monoclonal antibodies tend to be unstable and may alter the structure of antibody produced or stop producing antibody altogether (Kohler, G., et al. *Proc. Natl. Acad. Sci* (*USA*) 77: 2197 (1980); Morrison, S. L., *J. Immunol.* 123: 793 (1979)). The cell line genome appears to alter itself in response to stimuli whose nature is not currently known, and this alteration may result in production of incorrect sequences. Third, both hybridoma and B cells inevitably produce certain antibodies in glycosylated form (Melchers, F., *Biochemistry*, 10: 653 (1971)) which, under some circumstances, may be undesirable. Fourth, production of both monoclonal and polyclonal antibodies is relatively expensive. Fifth, and perhaps most important, production by current techniques (either by hybridoma or by B cell response) does not permit manipulation of the genome so as to produce antibodies with more effective design components than those normally elicited in response to antigens from the mature B cell in situ. The antibodies of the present invention do not suffer from the

foregoing drawbacks, and, furthermore, offer the opportunity to provide molecules of superior design.

Even those immunoglobulins which lack the specificity of antibodies are useful, although over a smaller spectrum of potential uses than the antibodies themselves. In presently understood applications, such immunoglobulins are helpful in proteins replacement therapy for globulin related anemia. In this context, an inability to bind to antigen is in fact helpful, as the therapeutic value of these proteins would be impaired by such functionality. At present, such non-specific antibodies are derivable in quantity only from myeloma cell cultures suitably induced. The present invention offers an alternative, more economical source. It also offers the opportunity of canceling out specificity by manipulating the four chains of the tetramer separately.

A.2 General Structure Characteristics

The basic immunoglobin structural unit in vertebrate systems is now well understood (Edelman, G. M., *Ann. N.Y. Acad. Sci*., 190: 5 (1971)). The units are composed of two identical light polypeptide chains of molecular weight approximately 23,000 daltons, and two identical heavy chains of molecular weight 53,000–70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket-the heavy-chains starting at the mouth of the Y and continuing through the divergent region as shown in FIG. 1. The "branch" portion, as there indicated, is designated the Fab region. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, with some subclasses among them, and the nature of this chain, as it has a long constant region, determines the "class"of the antibody as IgG, IgM, IgA, IgD, or IgE. Light chains are classified-as either kappa or lambda. Each heavy chain class can be prepared with either kappa or lambda light chain. The light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells. However, if non-covalent association of the chains can be effected in the correct geometry, the aggregate will still be capable of reaction with antigen, or of utility as a protein supplement as a non-specific immunoglobulin.

The amino acid sequence runs from the N-terminal end at the top of the Y to the C-terminal end at the bottom of each chain. At the N-terminal end is a variable region which is specific for the antigen which elicited it, and is approximately 100 amino acids in length, there being slight variations between light and heavy chain and from antibody to antibody. The variable region is linked in each chain to a constant region which extends the remaining length of the chain. Linkage is seen, at the genomic level, as occurring through a linking sequence known currently as the "J" region in the light chain gene, which encodes about 12 amino acids, and as a combination of "D" region and "J" region in the heavy chain gene, which together encode approximately 25 amino acids.

The remaining portions of the chain are referred to as constant regions and within a particular class do not to vary with the specificity of the antibody (i.e., the antigen eliciting it),

As stated above, there are five known major classes of constant regions which determine the class of the immunoglobulin molecule (IgG, IgM, IgA, IgD, and IgE corresponding to γ, μ, α, δ, and ϵ heavy chain constant regions). The constant region or class determines subsequent effector function of the antibody, including activation of complement (Kabat, E. A., *Structural Concepts in Immunology and Immunochemistry*, 2nd Ed., p. 413–436, Holt, Rinehart Winston (1976)), and other cellular responses (Andrews, D. W., et al., *Clinical Immunobiology* pp 1–18, W. B. Sanders (1980); Kohl, S., et al., *Immunology*, 48: 187 (1983)); while the variable region determines the antigen with which it will react.

B. Recombinant DNA Technology

Recombinant DNA technology has reached sufficient sophistication that it includes a repertoire of techniques for cloning and expression of gene sequences. Various DNA sequences can be recombined with some facility, creating new DNA entities capable of producing heterologous protein product in transformed microbes and cell cultures. The general means and methods for the in vitro ligation of various blunt ended or "sticky" ended fragments of DNA, for producing expression vectors, and for transforming organisms are now in hand.

DNA recombination of the essential elements (i.e., an origin of replication, one or more phenotypic selection characteristics, expression control sequence, heterologous gene insert and remainder vector) generally is performed outside the host cell. The resulting recombinant replicable expression vector, or plasmid, is introduced into cells by transformation and large quantities of the recombinant vehicle is obtained by growing the transformants. Where the gene is properly inserted with reference to portions which govern the transcription and translation of the encoded DNA message, the resulting expression vector is useful to produce the polypeptide sequence for which the inserted gene codes, a process referred to as "expression." The resulting product may be obtained by lysis, if necessary, of the host cell and recovery of the product by appropriate purifications from other proteins.

In practice, the use of recombinant DNA technology can express entirely heterologous polypeptides—so-called direct expression—or alternatively may express a heterologous polypeptide fused to a portion of the amino acid sequence of a homologous polypeptide. In the latter cases, the intended bioactive product is sometimes rendered bioinactive within the fused, homologous/heterologous polypeptide until it is cleaved in an extracellular environment.

The art of maintaining cell or tissue cultures as well as microbial systems for studying genetics and cell physiology is well established. Means and methods are available for maintaining permanent cell lines, prepared by successive serial transfers from isolated cells. For use in research, such cell lines are maintained on a solid support in liquid medium, or by growth in suspension containing support nutriments. Scale-up for large preparations seems to pose only mechanical problems.

SUMMARY OF THE INVENTION

The invention relates to antibodies and to non-specific immunoglobulins (NSIs) formed by recombinant techniques using suitable host cell cultures. These antibodies and NSIs can be readily prepared in pure "monoclonal" form. They can be manipulated at the genomic level to produce chimeras of variants which draw their homology from species which differ from each other. They can also be manipulated at the protein level, since all four chains do not need to be produced by the same cell. Thus, there are a number of "types" of immunoglobulins encompassed by the invention.

First, immunoglobulins, particularly antibodies, are produced using recombinant techniques which mimic the amino acid sequence of naturally occurring antibodies produced by either mammalian B cells in situ, or by B cells fused with suitable immortalizing tumor lines, i.e., hybridomas.

Second, the methods of this invention produce, and the invention is directed to, immunoglobulins which comprise polypeptides not hitherto found associated with each other in nature. Such reassembly is particularly useful in producing "hybrid" antibodies capable of binding more than one antigen; and in producing "composite" immunoglobulins wherein heavy and light chains of different origins essentially damp out specificity. Third, by genetic manipulation, "chimeric" antibodies can be formed wherein, for example, the variable regions correspond to the amino acid sequence from one mammalian model system, whereas the constant region mimics the amino acid sequence of another. Again, the derivation of these two mimicked sequences may be from different species. Fourth, also by genetic manipulation, "altered" antibodies with improved specificity and other characteristics can be formed.

Two other types of immunoglobulin-like moieties may be produced: "univalent" antibodies, which are useful as homing carriers to target tissues, and "Fab proteins" which include only the "Fab" region of an immunoglobulin molecule i.e., the branches of the "Y". These univalent antibodies and Fab fragments may also be "mammalian" i.e., mimic mammalian amino acid sequences; novel assemblies of mammalian chains, or chimeric, where for example, the constant and variable sequence patterns may be of different origin. Finally, either the light chain or heavy chain alone, or portions, thereof, produced by recombinant techniques are included in the invention and may be mammalian or chimeric.

In other aspects, the invention is directed to DNA which encodes the aforementioned NSIs, antibodies, and portions thereof, as well as expression vectors or plasmids capable of effecting the production of such immunoglobulins in suitable host cells. It includes the host cells and cell cultures which result from transformation with these vectors. Finally, the invention is directed to methods of producing these NSIs and antibodies, and the DNA sequences, plasmids, and transformed cells intermediate to them.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a representation of the general structure of immunoglobulins.

FIGS. **2**A–B shows the detailed sequence of the cDNA insert of pK17G4 which encodes kappa anti CEA chain.

FIG. **3** shows the coding sequence of the fragment shown in FIG. **2**, along with the corresponding amino acid sequence.

FIGS. **4**A–C shows the combined detailed sequence of the cDNA inserts of pγ298 and pγ11 which encode gamma anti CEA chain.

FIGS. **5**A–B shows the corresponding amino acid sequence encoded by the fragment in FIG. **4**.

FIGS. **6** and **7** outline the construction of expression vectors for kappa and gamma anti-CEA chains respectively.

FIGS. **8**A, **8**B, and **8**C show the results of sizing gels run on, extracts of *E. coli* expressing the genes for gamma chain, kappa chain, and both kappa and gamma chains respectively.

FIG. **9** shows the results of western blots of extracts of cells transformed as those in FIGS. **8**.

FIG. **10** shows a standard curve for ELISA assay of anti CEA activity.

FIGS. **11** and **12** show the construction of a plasmid for expression of the gene encoding a chimeric heavy chain.

FIG. **13** shows the construction of a plasmid for expression of the gene encoding the Fab region of heavy chain.

DETAILED DESCRIPTION

A. Definitions

As used herein, "antibodies" refers to tetramers or aggregates thereof which have specific immunoreactive activity, comprising light and heavy chains usually aggregated in the "Y" configuration of FIG. **1**, with or without covalent linkage between them; "immunoglobulins" refers to such assemblies whether or not specific immunoreactive activity is a property. "Non-specific immunoglobulin" ("NSI") means those immunoglobulins which do not possess specificity—i.e., those which are not antibodies.

"Mammalian antibodies" refers to antibodies wherein the amino acid sequences of the chains are homologous with those sequences found in antibodies produced by mammalian systems, either in situ, or in hybridomas. These antibodies mimic antibodies which are otherwise capable of being generated, although in impure form, in these traditional systems.

"Hybrid antibodies" refers to antibodies wherein chains are separately homologous with referenced mammalian antibody chains and represent novel assemblies of them, so that two different antigens are precipitable by the tetramer. In hybrid antibodies, one pair of heavy and light chain is homologous to antibodies raised against one antigen, while the other pair of heavy and light chain is homologous to those raised against another antigen. This results in the property of "divalence" i.e., ability to bind two antigens simultaneously. Such hybrids may, of course, also be formed using chimeric chains, as set forth below.

"Composite" immunoglobulins means those wherein the heavy and light chains mimic those of different species origins or specificities, and the resultant is thus likely to be a non-specific immunoglobulin (NSI), i.e.—lacking in antibody character.

"Chimeric antibodies" refers to those antibodies wherein one portion of each of the amino acid sequences of heavy and light chains is homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular class, while the remaining segment of the chains is homologous to corresponding sequences in another. Typically, in these chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals while the constant portions are homologous to the sequences in antibodies derived from another. One clear advantage to such chimeric forms is that, for example, the variable regions can conveniently be derived from presently known sources using readily available hybridomas or B cells from non human host organisms in combination with constant regions derived from, for example, human cell preparations. While the variable region has the advantage of ease of preparation, and the specificity is not affected by its source, the constant region being human, is less likely to elicit an immune response from a human subject when the antibodies are injected than would the constant region from a non-human source.

However, the definition is not limited to this particular example. It includes any antibody in which either or both of the heavy or light chains are composed of combinations of sequences mimicking the sequences in antibodies of different sources, whether these sources be differing classes, differing antigen responses, or differing species of origin and whether or not the fusion point is At the variable/constant boundary. Thus, it is possible to produce antibodies in which neither the constant nor the variable region mimic known antibody sequences. It then becomes possible, for example, to construct antibodies whose variable region has a higher

specific affinity for a particular antigen, or whose constant region can elicit enhanced complement fixation or to make other improvements in properties possessed by a particular constant region.

"Altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a mammalian or other vertebrate antibody. Because of the relevance of recombinant DNA techniques to this invention, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the constant region. Changes in the constant region will, in general, be made in order to improve the cellular process characteristics, such as complement fixation, interaction with membranes, and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics. The antibody can also be engineered so as to aid the specific delivery of a toxic agent according to the "magic bullet" concept. Alterations, can be made by standard recombinant techniques and also by oligonucleotide-directed mutagenesis techniques (Dalbadie-McFarland, et al *Proc. Natl. Acad. Sci.* (*USA*), 79:6.409 (1982)).

"Univalent antibodies" refers to aggregations which comprise a heavy chain/light chain dimer bound to the Fc (or stem) region of a second heavy chain. Such antibodies are specific for antigen but have the additional desirable property of targeting tissues with specific antigenic surfaces, without causing its antigenic effectiveness to be impaired—i.e., there is no antigenic modulation. This phenomenon and the property of univalent antibodies in this regard is set forth in Glennie, M. J., et al., *Nature*, 295: 712 (1982). Univalent antibodies have heretofore been formed by proteolysis.

"Fab" region refers to those portions of the chains which are roughly equivalent, or analogous, to the sequences which comprise the Y branch portions of the heavy chain and to the light chain in its entirety, and which collectively (in aggregates) have been shown to exhibit antibody activity. "Fab protein", which protein is one of the aspects of the invention, includes aggregates of one heavy and one light chain (commonly known as Fab'), as well as tetramers which correspond to the two branch segments of the antibody Y, (commonly known as $F(ab)_2$), whether any of the above are covalently or non-covalently aggregated, so long as the aggregation is capable of selectively reacting with a particular antigen or antigen family. Fab antibodies have, as have univalent ones, been formed heretofore by proteolysis, and share the property of not eliciting antigen modulation on target tissues. However, as they lack the "effector" Fc: portion they cannot effect, for example, lysis of the target cell by macrophages.

"Fab protein" has similar subsets according to the definition of the present invention as does the general term "antibodies" or "immunoglobulins". Thus, "mammalian" Fab protein, "hybrid" Fab protein "chimeric" Fab and "altered" Fab protein are defined analogously to the corresponding definitions set forth in the previous paragraphs for the various types of antibodies.

Individual heavy or light chains may of course be "mammalian", "chimeric" or "altered" in accordance with the above. As will become apparent from the detailed description of the invention, it is possible, using the techniques disclosed to prepare other combinations of the four-peptide chain aggregates, besides those specifically defined, such as hybrid antibodies containing chimeric light and mammalian heavy chains, hybrid Fab proteins containing chimeric Fab proteins of heavy chains associated with mammalian light chains, and so forth.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, i.e., the coding sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. A useful, but not a necessary, element of an effective expression vector is a marker encoding sequence—i.e. a sequence encoding a protein which results in a phenotypic property (e.g. tetracycline resistance) of the cells containing the protein which permits those cells to be readily identified. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified contained DNA code is included in this term, as it is applied to the specified sequence. As at present, such vectors are frequently in the form of plasmids, thus "plasmid" and "expression vector" are often used interchangeably. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which may, from time to time become known in the art.

"Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a recombinant host cell is by virtue of this transformation, rather than in such lesser amounts, or more commonly, in such less than detectable amounts, as would be produced by the untransformed host.

In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of antibody from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

B. Host Cell Cultures and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In-general, of course, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATTC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F^-, λ^-, prototrophic, ATTC No. 27325), bacilli such as *Bacillus subtilus*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various Pseudomonas species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., *Gene* 2: 95 (1977)). PBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial

plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems Chang et al, *Nature* 275: 615 (1978); Itakura, et al, *Science* 198: 1056 (1977); (Goeddel et al, *Nature* 281: 544 (1979)) and a tryptophan (trp) promoter system (Goeddel, et al, *Nucleic kids Res.*, 8: 4057 (1980); EPO Appl Publ No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized., and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist, et al, Cell 20: 269 (1980)).

In addition to prokaryates, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (Stinchcomb et al, *Nature*, 282: 39 (1979); Kingsman et al, *Gene*, 7: 141 (1979); Tschemper, et al, *Gene*, 10: 157 (1980)) is commonly used. This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85: 12 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.*, 255: 2073 (1980)) or other glycolytic enzymes (Hess, et al, *J. Adv. Enzyme Reg.*, 7: 149 (1968); Holland, et al, *Biochemistry*, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization (Holland, ibid.). Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers, et al, *Nature*, 273: 113 (1978)) incorporated herein by reference. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g. Polyoma, Adeno, VSV, BPV, etc.) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

It will be understood that this invention, although described herein in terms of a preferred embodiment, should not be construed as limited to those host cells, vectors and expression systems exemplified.

C. Methods Employed

C.1 Transformation

If cells without formidable cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology*, 52: 546 (1978). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F. N. et al *Proc. Natl. Acad. Sci.* (*USA*) 69: 2110 (1972).

C.2 Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. The methods employed are not dependent on the DNA source, or intended host.

Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1 μg plasmid or DNA fragments is used with about 1 unit of enzyme in about 20 μl of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about 1 hour at 37° C. are workable. After incubations, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation is treated for 15 minutes at 15° with 10 units of *E. coli* DNA Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel, D., et al, *Nucleic Acids Res.*, 8: 4057 (1980) incorporated herein by reference.

For ligation, approximately equimolar amounts of the desired components, suitably end tailored to provide correct matching are treated with about 10 units T4 DNA ligase per 0.5 μg DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

In the examples described below correct ligations for plasmid construction are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with the ligation mixture. Successful transformants were selected by ampicillin or tetracycline resistance depending on the mode of plasmid construction. Plasmids from the transformants were then prepared, analyzed by restriction and/or sequenced by the method of Messing, et al, *Nucleic Acids Res.,* 9:309 (1981) or by the method of Maxam, et al, *Methods in Enzymology,* 65:499 (1980).

D. Outline of Procedures

D.1 Mammalian Antibodies

The first type of antibody which forms a part of this invention, and is prepared by the methods thereof, is "mammalian antibody"-one wherein the heavy and light chains mimic the amino acid sequences of an antibody otherwise produced by a mature mammalian B lymphocyte either in situ or when fused with an immortalized cell as part of a hybridoma culture. In outline, these antibodies are produced as follows:

Messenger RNA coding for heavy or light chain is isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation, and the use of oligo-dT cellulose chromatography to segregate the poly-A mRNA. The poly-A mRNA may, further, be fractionated to obtain sequences of sufficient size to code for the amino acid sequences in the light or heavy chain of the desired antibody as the case may be.

A cDNA library is then prepared from the mixture of mRNA using a suitable primer, preferably a nucleic acid sequence which is characteristic of the desired cDNA. Such a primer may be hypothesized and synthesized based on the amino acid sequence of the antibody if the sequence is known. In the alternative cDNA from unfractionated poly-A mRNA from a cell line producing the desired antibody or poly-dT may also be used. The resulting cDNA is optionally size fractionated on polyacrylamide gel and then extended with, for example, dC residues for annealing with pBR322 or other suitable cloning vector which has been cleaved by a suitable restriction enzyme, such as Pst I, and extended with dG residues. Alternative means of forming cloning vectors containing the cDNA using other tails and other cloning vector remainder may, of course, also be used but the foregoing is a standard and preferable choice. A suitable host cell strain, typically *E. coli,* is transformed with the annealed cloning vectors, and the successful transformants identified by means of, for example, tetracycline resistance or other phenotypic characteristic residing on the cloning vector plasmid.

Successful transformants are picked and transferred to microtiter dishes or other support for further growth and preservation. Nitrocellulose filter imprints of these growing cultures are then probed with suitable nucleotide sequences containing bases known to be complementary to desired sequences in the cDNA. Several types of probe may be used, preferably synthetic single stranded DNA sequences labeled by kinasing with ATP^{32}. The cells fixed to the nitrocellulose filter are lysed, the DNA denatured, and then fixed before reaction with kinased probe. Clones which successfully hybridize are detected by contact with a photoplate, then plasmids from the growing colonies isolated and sequenced by means known in the art to verify that the desired portions of the gene are present.

The desired gene fragments are excised and tailored to assure appropriate reading frame with the control segments when inserted into suitable expression vectors. Typically, nucleotides are added to the 5' end to include a start signal and a suitably positioned restriction endonuclease site.

The tailored gene sequence is then positioned in a vector which contains a promoter in reading frame with the gene and compatible with the proposed host cell. A number of plasmids such as those described in U.S. patent application Ser. Nos. 307,473; 291,892; and 305,657 (EPO Publ. Nos. 0036776; 0048970 and 0051873) have been described which already contain the appropriate promoters, control sequences, ribosome binding sites, and transcription termination sites, as well as convenient markers.

In the present invention, the gene coding for the light chain and that coding for the heaving chain are recovered separately by the procedures outlined above. Thus then may be inserted into separate expression plasmids, or together in the same plasmid, so long as each is under suitable promoter and translation control.

The expression vectors constructed above are then used to transform suitable cells. The light and heavy chains may be transformed into separate cell cultures, either of the same or of differing species; separate plasmids for light and heavy chain may be used to co-transform a single cell culture, or, finally, a single expression plasmid containing both genes and capable of expressing the genes for both light and heavy chain may be transformed into a single cell culture.

Regardless of which of the three foregoing options is chosen, the cells are grown under conditions appropriate to the production of the desired protein. Such conditions are primarily mandated by the type of promoter and control systems used in the expression vector, rather than by the nature of the desired protein. The protein thus produced is then recovered from the cell culture by methods known in the art, but choice of which is necessarily dependent on the form in which the protein is expressed. For example, it is common for mature heterologous proteins expressed in *E. coli* to be deposited within the cells as insoluble particles which require cell lysis and solubilization in denaturant to permit recovery. On the other hand, proteins under proper synthesis circumstances, in yeast and bacterial strains, can be secreted into the medium (yeast and gram positive bacteria) or into the periplasmic space (gram negative bacteria) allowing recovery by less drastic procedures. Tissue culture cells as hosts also appear, in general, to permit reasonably facile recovery of heterologous proteins.

When heavy and light chain are coexpressed in the same host, the isolation procedure is designed so as to recover reconstituted antibody. This can be accomplished in vitro as described below, or might be possible in vivo in a microorganism which secretes the IgG chains out of the reducing environment of the cytoplasm. A more detailed description is given in D.2, below.

D.2 Chain Recombination Techniques

The ability of the method of the invention to produce heavy and light chains or portions thereof, in isolation from each other offers the opportunity to obtain unique and unprecedented assemblies of immunoglobulins, Fab regions, and univalent antibodies. Such preparations require the use of techniques to reassemble isolated chains. Such means are known in the art, and it is, thus, appropriate to review them here.

While single chain disulfide bond containing proteins have been reduced and reoxidized to regenerate in high yield

native structure and activity (Freedman, R. B., et al. In *Enzymology of Post Translational Modification of Proteins,* I: 157–212 (1980) Academic Press, NY.), proteins which consist of discontinuous polypeptide chains held together by disulfide bonds are more difficult to reconstruct in vitro after reductive cleavage. Insulin, a cameo case, has received much experimental attention over the years, and can now be reconstructed so efficiently that an industrial process has been built around it (Chance, R. E., et al., In *Peptides: Proceedings of the Seventh Annual American Peptide Symposium* (Rich, D. H. and Gross. E., eds.) 721–728, Pierce Chemical Co., Rockford, Ill. (1981)).

Immunoglobulin has proved a more difficult problem than insulin. The tetramer is stabilized intra and intermolecularly by 15 or more disulfide bonds. It has been possible to recombine heavy and light chains, disrupted by cleavage of only the interchain disulfides, to regain antibody activity even without restoration of the inter-chain disulfides (Edelman, G. M., et al., *Proc. Natl. Acad. Sci. (USA)* 50: 753 (1963)). In addition, active fragments of IgG formed by proteolysis (Fab fragments of ~50,000 MW) can be split into their fully reduced heavy chain and light chain components and fairly efficiently reconstructed to give active antibody (Haber, E., *Proc. Natl. Acad. Sci. (USA)* 52: 1099 (1964); Whitney, P. L., et al., *Proc. Natl. Acad. Sci. (USA)* 53: 524 (1965)). Attempts to reconstitute active antibody from fully reduced native IgG have been largely unsuccessful, presumably due to insolubility of the reduced chains and of side products or intermediates in the refolding pathway (see discussion in Freedman, M. H., et al., *J. Biol. Chem.* 241: 5225 (1966)). If, however, the immunoglobulin is randomly modified by polyalanylation of its lysines before complete reduction, the separated chains have the ability to recover antigen-combining activity upon reoxidation (ibid).

A particularly suitable method for immunoglobulin reconstitution is derivable from the now classical insulin recombination studies, wherein starting material was prepared by oxidative sulfitolysis, thus generating thiol-labile S-sulfonate groups at all cysteines in the protein, non-reductively breaking disulfides (Chance et al. (supra)). Oxidative sulfitolysis is a mild disulfide cleavage reaction (Means, G. E., et al., *Chemical Modification of Proteins,* Holden-Day, San Francisco (1971)) which is sometimes more gentle than reduction, and which generates derivatives which are stable until exposed to mild reducing agent at which time disulfide reformation can occur via thiol-disulfide interchange. In the present invention the heavy and light chain S-sulfonates generated by oxidative sulfitolysis were reconstituted utilizing both air oxidation and thiol-disulfide interchange to drive disulfide bond formation. The general procedure is set forth in detail in U.S. Ser. No. 452,187, filed Dec. 22, 1982 (EPO Appln. No. 83.307840.5), incorporated herein by reference.

D.3 Variants Permitted by Recombinant Technology

Using the techniques described in paragraphs D.1 and D.2, additional operations which were utilized to gain efficient production of mammalian antibody can be varied in quite straightforward and simple ways to produce a great variety of modifications of this basic antibody form. These variations are inherent in the use of recombinant technology, which permits modification at a genetic level of amino acid sequences in normally encountered mammalian immunoglobulin chains, and the great power of this approach lies in its ability to achieve these variations, as well as in its potential for economic and specific production of desired scarce, and often contaminated, molecules. The variations also inhere in the ability to isolate production of individual chains, and thus create novel assemblies.

Briefly, since genetic manipulations permit reconstruction of genomic material in the process of construction of expression vectors, such reconstruction can be manipulated to produce new coding sequences for the components of "natural" antibodies or immunoglobulins. As discussed in further detail below, the coding sequence for a mammalian heavy chain may not be derived entirely from a single source or single species, but portions of a sequence can be recovered by the techniques described in D.1 from differing pools of mRNA, such as murine-murine hybridomas, human-murine hybridomas, or B cells differentiated in response to a series of antigen challenges. The desired portions of the sequences in each case can be recovered using the probe and analysis techniques described in D.1, and recombined in an expression vector using the same ligation procedures as would be employed for portions of the same model sequence. Such chimeric chains can be constructed of any desired length; hence, for example, a complete heavy chain can be constructed, or only sequence for the Fab region thereof.

The additional area of flexibility which arises from the use of recombinant techniques results from the power to produce heavy and light chains or fragments thereof in separate cultures or of unique combinations of heavy and light chain in the same culture, and to prevent reconstitution of the antibody or immunoglobulin aggregation until the suitable components are assembled. Thus, while normal antibody production results automatically in the formation of "mammalian antibodies" because the light and heavy chain portions are constructed in response to a particular determinant in the same cell, the methods of the present invention present the opportunity to assemble entirely new mixtures. Somewhat limited quantities of "hybrid" antibodies have been produced by "quadromas" i.e., fusions of two hybridoma cell cultures which permit random assemblies of the heavy and light chains so produced.

The present invention permits a more controlled assembly of desired chains, either by mixing the desired chains in vitro, or by transforming the same culture with the coding sequences for the desired chains.

D.4 Composite Immunoglobulins

The foregoing procedure, which describes in detail the recombinant production of mammalian antibodies is employed with some modifications to construct the remaining types of antibodies or NSIs encompassed by the present invention. To prepare the particular embodiment of composite non-specific immunoglobulin wherein the homology of the chains corresponds to the sequences of immunoglobulins of different specificities, it is of course, only necessary to prepare the heavy and light chains in separate cultures and reassemble them as desired.

For example, in order to make an anti-CEA light chain/antihepatitis heavy chain composite antibody, a suitable source for the mRNA used as a template for the light chain clone would comprise, for instance, the anti CEA producing cell line of paragraph E.1. The mRNA corresponding to heavy chain would be derived from B cells raised in response to hepatitis infection or from hybridoma in which the B cell was of this origin. It is clear that such composites can be assembled using the methods of the invention almost at will, and are limited only by available sources of mRNA suitable for use as templates for the respective chains. All other features of the process are similar to those described above.

D.5 Hybrid Antibodies

Hybrid antibodies are particularly useful as they are capable of simultaneous reaction with more than one antigen. Pairs of heavy and light chains corresponding to chains

of antibodies for different antigens, such as those set forth in paragraph D.4 are prepared in four separate cultures, thus preventing premature assembly of the tetramer. Subsequent mixing of the four separately prepared peptides then permits assembly into the desired tetramers. While random aggregation may lead to the formation of considerable undesired product, that portion of the product in which homologous light and heavy chains are bound to each other and mismatched to another pair gives the desired hybrid antibody.

D.6 Chimeric Antibodies

For construction of chimeric antibodies (wherein, for example, the variable sequences are separately derived from the constant sequences) the procedures of paragraph C.1 and D.2 are again applicable with appropriate additions and modifications. A preferred procedure is to recover desired portions of the genes encoding for parts of the heavy and light chains from suitable, differing, sources and then to religate these fragments using restriction endonucleases to reconstruct the gene coding for each chain.

For example, in a particularly preferred chimeric construction, portions of the heavy chain gene and of the light chain gene which encode the variable sequences of antibodies produced by a murine hybridoma culture are recovered and closed from this culture and gene fragments encoding the constant regions of the heavy and light chains for human antibodies recovered and cloned from, for example, human myeloma cells. Suitable restriction enzymes may then be used to ligate the variable portions of the mouse gene to the constant regions of the human gene for each of the two chains. The chimeric chains are produced as set forth in D.1, aggregated as set forth in D.2 and used in the same manner as the non-chimeric forms. Of course, any splice point in the chains can be chosen.

D.7 Altered Antibodies

Altered antibodies present, in essence, an extension of chimeric ones. Again, the techniques of D.1 and D.2 are applicable; however, rather than splicing portions of the chain(s), suitable amino acid alterations, deletions or additions are made using available techniques such as mutagenesis (supra). For example, genes which encode antibodies having diminished complement fixation properties, or which have enhanced metal binding capacities are prepared using such techniques. The latter type may, for example, take advantage of the known gene sequence encoding metallothionein II (Karin, M., et al., *Nature,* 299: 797 (1982)). The chelating properties of this molecular fragment are useful in carrying heavy metals to tumor sites as an aid in tumor imaging (Scheinberg, D. A., et al., *Science,* 215: 19 (1982).

D.8 Univalent Antibodies

In another preferred embodiment, antibodies are formed which comprise one heavy and light chain pair coupled with the Fc region of a third (heavy) chain. These antibodies have a particularly useful property. They can, like ordinary antibodies, be used to target antigenic surfaces of tissues, such as tumors, but, unlike ordinary antibodies, they do not cause the antigenic surfaces of the target tissue to retreat and become non-receptive. Ordinary antibody use results in aggregation and subsequent inactivation, for several hours, of such surface antigens.

The method of construction of univalent antibodies is a straightforward application of the invention. The gene for heavy chain of the desired Fc region is cleaved by restriction enzymes, and only that portion coding for the desired Fc region expressed. This portion is then bound using the technique of D.2 to separately produced heavy chain the desired pairs separated from heavy/heavy and Fc/Fc combinations, and separately produced light chain added. Pre-binding of the two heavy chain portions thus diminishes the probability of formation of ordinary antibody.

D.9 Fab Protein

Similarly, it is not necessary to include the entire gene for the heavy chain portion. All of the aforementioned variations can be superimposed on a procedure for Fab protein production and the overall procedure differs only in that the portion of the heavy chain coding for the amino terminal **220** amino acids is employed in the appropriate expression vector.

E. Specific Examples of Preferred Embodiments

The invention has been described above in general terms and there follow several specific examples of embodiments which set forth details of experimental procedure in producing the desired antibodies. Example E.1 sets forth the general procedure for preparing anti CEA antibody components, i.e. for a "mammalian antibody". Example E.3 sets forth the procedure for reconstitution and thus is applicable to preparation of mammalian, composite, hybrid and chimeric immunoglobulins, and Fab proteins and univalent antibodies. Example E.4 sets forth the procedure for tailoring the heavy or light chain so that the variable and constant regions may be derived from different sources. Example E.5 sets forth the method of obtaining a shortened heavy chain genome which permits the production of the Fab regions and, in an analogous manner, Fc region.

The examples set forth below are included for illustrative purposes and do not limit the scope of the invention.

E.1 Construction of Expression Vectors for Murine anti-CEA Antibody Chains and Peptide Synthesis

Carcinoembryonic antigen (CEA) is associated with the surface of certain tumor cells of human origin (Gold, P., et al., *J. Exp. Med.,* 122: 467 (1965)). Antibodies which bind to CEA (anti-CEA antibodies) are useful in early detection of these tumors (Van Nagell, T. R., et al., *Cancer Res.* 40: 502 (1980)), and have the potential for use in treatment of those human tumors which appear to support CEA at their surfaces. A mouse hybridoma cell line which secretes anti-CEA antibodies of the $Ig\gamma_1$ class, CEA.66-E3, has been prepared as described by Wagener, C, et al., *J. Immunol.* (in press) which is incorporated herein by reference, and was used as mRNA source. The production of anti CEA antibodies by this cell line was determined. The N-terminal sequences of the antibodies produced by these cells was compared with those of monoclonal anti CEA as follows. Purified IgG was treated with PCAse (Podell, D. N., et al., BBRC 81: 176 (1978)), and then dissociated in 6M guanidine hydrochloride, 10 mM 2-mercaptoethanol (1.0 mg of immunoglobulin, 5 min, 100° C. water bath). The dissociated chains were separated on a Waters Associates alkyl phenyl column using a linear gradient from 100 percent A (0.1 percent TFA-water) to 90 percent B (TFA/H_2O/MeCN 0.1/9.9/90) at a flow rate of 0.8 ml/min. Three major peaks were eluted and analyzed on SDS gels by silver staining. The first two peaks were pure light chain (MW 25,000 daltons), the third peak showed a (7:3) mixture of heavy and light chain. 1.2 nmoles of light chain were sequenced by the method of Shively, J. E., *Methods in Enzymology,* 79: 31 (1981), with an NH_2-terminal yield of 0.4 nmoles. A mixture of heavy and light chains (3 nmoles) was also sequenced, and sequence of light chain was deducted from the double sequence to yield the sequence of the heavy chain.

In the description which follows, isolation and expression of the genes for the heavy and light chains for anti CEA antibody produced by CEA.66-E3 are described. As the constant regions of these chains belong to the gamma and kappa families, respectively, "light chain" and "kappa

chain", and "heavy chain" and "gamma chain", respectively, are used interchangeably below.

E.1.1 Isolation of Messenger RNA for Anti CEA Light and Heavy (Kappa and Gamma) Chains

Total RNA from CEA.66-E3 cells was extracted essentially as reported by Lynch et al, *Virology,* 98: 251 (1979). Cells were pelleted by centrifugation and approximately 1 g portions of pellet resuspended in 10 ml of 10 mM NaCl, 10 mM Tris HCl (pH 7.4), 1.5 mM $MgCl_2$. The resuspended cells were lysed by addition of non-ionic detergent NP-40 to a final concentration of 1 percent, and nuclei removed by centrifugation. After addition of SDS (pH 7.4) to 1 percent final concentration, the supernatant was extracted twice with 3 ml portions of phenol (redistilled)/chloroform: isoamyl alcohol 25:1 at 4° C. The aqueous phase was made 0.2 M in NaCl and total RNA was precipitated by addition of two volumes of 100 percent ethanol and overnight storage at −20° C. After centrifugation, polyA mRNA was purified from total RNA by oligo-dT cellulose chromatography as described by Aviv and Leder, *Proc. Nat'l, Acad. Sci. (USA),* 69: 1408 (19672), 142 μg of polyA mRNA was obtained from 1 g cells.

E.1.2 Preparation of *E. coli* Colony Library Containing Plasmids with Heavy and Light DNA Sequence Inserts

5 μg of the unfractionated polyA mRNA prepared in paragraph E.1.1 was used as template for oligo-dT primed preparation of double-stranded (ds) cDNA by standard procedures as described by Goeddel et al., *Nature* 281: 544 (1979) and Wickens et al., *J. Biol. Chem.* 253: 2483 (1978) incorporated herein by reference. The cDNA was size fractionated by 6 percent polyacrylamide gel electrophoresis and 124 ng of ds cDNA greater than 600 base pairs in length was recovered by electroelution. A 20 ng portion of ds cDNA was extended with deoxy C residues using terminal deoxynucleotidyl transferase as described in Chang et al., *Nature* 275: 617 (1978) incorporated herein by reference, and annealed with 200 ng of the plasmid pBR322 (Bolivar et al., *Gene* 2: 95 (1977)) which had been cleaved with Pst I and tailed with deoxy G. Each annealed mixture was then transformed into *E. coli* K12 strain 294 (ATCC No. 31446). Approximately 8500 ampicillin sensitive, tetracycline resistant transformants were obtained.

E.1.3 Preparation of Synthetic Probes

The 14mer, 5' GGTGGGAAGATGGA 3' complementary to the coding sequence of constant region for mouse MOPC21 kappa chain which begins 25 basepairs 3' of the variable region DNA sequence was used as kappa chain probe. A 15 mer, 5' GACCAGGCATCCCAG 3', complementary to a coding sequence located 72 basepairs 3' of the variable region DNA sequence for mouse MOPC21 gamma chain was used to probe gamma chain gene.

Both probes were synthesized by the phosphotriester method described in German Offenlegungschrift 2644432, incorporated herein by reference, and made radioactive by kinasing as follows: 250 ng of deoxyoligonucleotide were combined in 25 μl of 60 mM Tris HCl (pH 8), 10 mM $MgCl_2$, 15 mM beta-mercaptoethanol, and 100 μCi (γ-^{32}P) ATP (Amersham, 5000 Ci/mMole). 5 units of T4 polynucleotide kinase were added and the reduction was allowed to proceed at 37° C. for 30 minutes and terminated by addition of EDTA to 20 mM.

E.1.4 Screening of Colony Library for Kappa or Gamma Chain Sequences

~2000 colonies prepared as described in paragraph E.1.2 were individually inoculated into wells of microtitre dishes containing LB (Miller, Experiments in Molecular Genetics, p. 431–3, Cold Spring Harbor, Lab., Cold Spring Harbor, N.Y. (1972))+5 μg/ml tetracycline and stored at −20° C. after addition of DMSO to 7 percent. Individual colonies from this library were transferred to duplicate sets of Schleicher and Schuell BA85/20 nitrocellulose filters and grown on agar plates containing LB+5 μg/ml tetracycline. After ~10 hours growth at 37° C. the colony filters were transferred to agar plates containing LB+5 μg/ml tetracycline and 12.5 μg/ml chloramphenicol and reincubated overnight at 37° C. The DNA from each colony was then denatured and fixed to the filter by a modification of the Grunstein-Hogness procedure as described in Grunstein et al., *Proc. Natl. Acad. Sci. (USA)* 72: 3961 (1975), incorporated herein by reference. Each filter was floated for 3 minutes on 0.5 N NaOH, 1.5 M NaCl to lyse the colonies and denature the DNA then neutralized by floating for 15 minutes on 3 M NaCl, 0.5 M Tris HCl (pH 7.5). The filters were then floated for an additional 15 minutes on 2XSSC, and subsequently baked for 2 hours in an 80° C. vacuum oven. The filters were prehybridized for ~2 hours at room temperature in 0.9 M NaCl, 1X Denhardts, 100 mM Tris HCl (pH 7.5), 5 mM Na-EDTA, 1 mM ATP, 1 M sodium phosphate (dibasic), 1 mM sodium pyrophosphate, 0.5 percent NP-40, and 200 μg/ml *E. coli* t-RNA, and hybridized in the same solution overnight, essentially as described by Wallace et al. *Nucleic Acids Research* 9: 879 (1981) using ~40×10^6 cpm of either the kinased kappa or gamma probe described above.

After extensive washing at 37° C. in 6X SSC, 0.1 percent SDS, the filters were exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens for 16–24 hours at −80° C. Approximately 20 colonies which hybridized with kappa chain probe and 20 which hybridized with gamma chain probe were characterized.

E.1.5 Characterization of Colonies which Hybridize to Kappa DNA Sequence Probe

Plasmid DNAs isolated from several different transformants which hybridized to kappa chain probe were cleaved with Pst I and fractionated by polyacrylamide gel electrophoresis (PAGE). This analysis demonstrated that a number of plasmid DNAs contained cDNA inserts large enough to encode full length kappa chain. The complete nucleotide sequence of the cDNA insert of one of these plasmids was determined by the dideoxynucleotide chain termination method as described by Smith, *Methods Enzymol.* 65, 560 (1980) incorporated herein by reference after subcloning restriction endonuclease cleavage fragments into M13 vectors (Messing et al., *Nucleic Acids Research* 9: 309 (1981). FIG. **2** shows the nucleotide sequence of the cDNA insert of pK17G4 and FIG. **3** shows the gene sequence with the corresponding amino acid sequence. Thus, the entire coding region of mouse anti-CEA kappa chain was isolated on this one large DNA fragment. The amino acid sequence of kappa chain, deduced from the nucleotide sequence of the pK17G4 cDNA insert, corresponds perfectly with the first 23 N-terminal amino acids of mature mouse anti-CEA kappa chain as determined by amino acid sequence analysis of purified mouse anti-CEA kappa chain. The coding region of pK17G4 contains 27 basepairs or 9 amino acids of the presequence and 642 basepairs or 214 amino acids of the mature protein. The mature unglycosylated protein (MW 24,553) has a variable region of 119 amino acids, including the J1 joining region of 12 amino acids, and a constant region of 107 amino acids. After the stop codon behind amino acids 215 begins 212 basepairs of 3' untranslated sequence up to the polyA addition. The kappa chain probe used to identify pK17G4 hybridizes to nucleotides 374–388 (FIG. **2**).

E.1.6 Characterization of Colonies which Hybridize to Gamma 1 DNA Probe

Plasmid DNA isolated from several transformants positive for hybridization with the heavy chain gamma 1 probe was subjected to Pst I restriction endonuclease analysis as described in E.1.5. Plasmid DNAs demonstrating the largest cDNA insert fragments were selected for further study. Nucleotide sequence coding for mouse heavy (gamma-1) chain, shows an NcoI restriction endonuclease cleavage site near the junction between variable and constant region. Selected plasmid DNAs were digested with both PstI and NcoI and sized on polyacrylamide. This analysis allowed identification of a number of plasmid DNAs that contain NcoI restriction endonuclease sites, although none that demonstrate cDNA insert fragments large enough to encode the entire coding region of mouse anti-CEA heavy chain.

In one plasmid isolated, p γ298 the cDNA insert of about 1300 bp contains sequence information for the 5' untranslated region, the signal sequence and the N-terminal portion of heavy chain. Because pγ298 did not encode the C-terminal sequence for mouse anti-CEA gamma 1 chain, plasmid DNA was isolated from other colonies and screened with PstI and NcoI. The C-terminal region of the cDNA insert of pγ11 was sequence and shown to contain the stop codon, 3' untranslated sequence and that portion of the coding sequence missing from p γ298.

FIG. **4** presents the entire nucleotide sequence of mouse anti-CEA heavy chain (as determined by the dideoxynucleotide chain termination method of Smith, *Methods Enzymol.,* 65: 560 (1980)) and FIG. **5** includes the translated sequence.

The amino acid sequence of gamma 1 (heavy chain) deduced from the nucleotide sequence of the pγ298 cDNA insert corresponds perfectly to the first 23 N-terminal amino acids of mature mouse anti-CEA gamma 1 chain as determined by amino acid sequence analysis of purified mouse anti-CEA gamma-1 chain. The coding region consists of 57 basepairs or 19 amino acids of presequences and 1346 basepairs or 447 amino acids of mature protein. The mature unglycosolated protein (MW 52,258) has a variable region of 135 amino acids, including a D region of 12 amino acids, and a J4 joining region of 13 amino acids. The constant region is 324 amino acids. After the stop codon behind amino acid 447 begins 96 bp of 3' untranslated sequences up to the polyA addition. The probe used to identify pγ298 and pγ11 hybridized to nucleotides 528–542 (FIG. **4**).

E.1.7 Construction of a Plasmid for Direct Expression of Mouse Mature Anti-CEA Kappa Chain Gene, pKCEAtrp207-1*

FIG. **6** illustrated the construction of pKCEAtrp207-1*

First, an intermediate plasmid pHGH207-1*, having a single trp promoter, was prepared as follows:

The plasmid pHGH 207 (described in U.S. patent application Ser. No. 307,473, filed Oct. 1, 1981 (EPO Publn. No. 0036776)) has a double lac promoter followed by the trp promoter, flanked by EcoR I sites and was used to prepare pHGH207-1. pHGH207 was digested with BamH 1, followed by partial digestion with EcoR I. The largest fragment, which contains the entire trp promoter, was isolated and ligated to the largest EcoR I- BamH I fragment from pBR322, and the ligation mixture used to transform *E. coli* 294. Tet^R Amp^R colonies were isolated, and most of them contained pHGH207-1. pHGH207-1* which lacks the EcoR1 site between the amp^R gene and the trp promoter, was obtained by partial digestion of pHGH207-1 with EcoR I, filling in the ends with Klenow and dNTPs, and religation.

5 μg of pHGH207-1* was digested with EcoRI, and the ends extended to blunt ends using 12 units of DNA Polymerase I in a 50 μl reaction containing 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4) and 1 mM in each dNTP at 37° C. for 1 hour, followed by extraction with phenol/$CHCl_3$ and precipitation with ethanol. The precipitated DNA was digested with BamH I, and the large vector fragment (fragment 1) purified using 5 percent polyacrylamide gel electrophoresis, electroelution, phenol/$CHCl_3$ extraction and ethanol precipitation.

The DNA was resuspended in 50 μl of 10 mM Tris pH 8, 1 mM EDTA and treated with 500 units Bacterial Alkaline Phosphatase (BAP) for 30' at 65° followed by phenol/$CHCl_3$ extraction and ethanol precipitation.

A DNA fragment containing part of the light chain sequence was prepared as follows: 7 μg of pH17G4 DNA was digested with Pst I and the kappa chain containing cDNA insert was isolated by 6 percent gel electrophoresis, and electroelution. After phenol/$CHCl_3$ extraction, ethanol precipitation and resuspension in water, this fragment was digested with Ava II. The 333 bp Pst I-Ava II DNA fragment was isolated and purified from a 6 percent polyacrylamide gel.

A 15 nucleotide DNA primer was synthesized by the phosphotriester method G. O. 2,644,432 (supra) and has the following sequence:

```
Met Asp Ile Val Met
5' ATG GAC ATT GTT ATG 3'
```

The 5' methionine serves as the initiation codon. 500 ng of this primer was phosphorylated at the 5' end with 10 units T4 DNA kinase in 20 μl reaction containing 0.5 mM ATP. ~200 ng of the Pst I-Ava II DNA fragment was mixed with the 20 μl of the phosphorylated primer, heated to 95° C. for 3 minutes and quick frozen in a dry-ice ethanol bath. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C. this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with Sau 3A. The reaction mixture was then electrophoresed on a 6 percent polyacrylamide gel and ~50 ng of the 182 basepair amino-terminal blunt-end to Sau 3A fragment (fragment 2) was obtained after electroelution.

100 ng of fragment 1 (supra) and 50 ng of fragment 2 were combined in 20 μl of 20 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 2.5 mM ATP and 1 unit of T4 DNA ligase. After overnight ligation at 14° C. the reaction was transformed into *E. coli* K12 strain 294. Restriction endonuclease digestion of plasmid DNA from a number of ampicillin resistant transformants indicated the proper construction and DNA sequence analysis proved the desired nucleotide sequence through the initiation codon of this new plasmid, pKCEAInt1 (FIG. **6**).

The remainder of the coding sequence of the kappa light chain gene was prepared as follows:

The Pst I cDNA insert fragment from 7 μg of K17G4 DNA was partially digested with Ava II and the Ava II cohesive ends were extended to blunt ends in a DNA Polymerase I large fragment reaction. Following 6 percent polyacrylamide gel electrophoresis the 686 basepair Pst I to blunt ended Ava II DNA fragment was isolated, purified and subjected to Hpa II restriction endonuclease digestion. The 497 basepair Hpa II to blunt ended Ava II DNA fragment (fragment 3) was isolated and purified after gel electrophoresis.

10 μg of pKCEAInt1 DNA was digested with Ava I, extended with DNA polymerase I large fragment, and

digested with Xba I. Both the large blunt ended Ava I to Xba I vector fragment and the small blunt ended Ava I to Xba I fragment were isolated and purified from a 6 percent polyacrylamide gel after electrophoresis. The large vector fragment (fragment 4) was treated with Bacterial Alkaline Phosphatase (BAP), and the small fragment was digested with Hpa II, electrophoresed on a 6 percent polyacrylamide and the 169 basepair Xba I-Hpa II DNA fragment (fragment 5) was purified. ~75 ng of fragment 4, ~50 ng of fragment 3 and ~50 ng of fragment 5 were combined in a T4 DNA ligase reaction and incubated overnight at 14°, and the reaction mixture transformed into *E. coli* K12 strain 294. Plasmid DNA from six ampicillin resistant transformants were analyzed by restriction endonuclease digestion. One plasmid DNA demonstrated the proper construction and was designated pKCEAInt2.

Final construction was effected by ligating the K-CEA fragment, including the trp promoter from pKCEAInt2 into pBR322(XAP). (pBR322(XAP) is prepared as described in U.S. application Ser. No. 452,227, filed Dec. 22, 1982; from pBR322 by deletion of the AvaI-PvuII fragment followed by ligation.)

The K-CEA fragment was prepared by treating pKCEAInt2 with Ava I, blunt ending with DNA polymerase I (Klenow fragment) in the presence of DNTPs, digestion with Pst I and isolation of the desired fragment by gel electrophoresis and electroelution.

The large vector fragment from pBR322(XAP) was prepared by successive treatment with EcoR I, blunt ending with polymerase, and redigestion with Pst I, followed by isolation of the large vector fragment by electrophoresis and electroelution.

The K-CEA and large vector fragments as prepared in the preceding paragraphs were ligated with T4 DNA ligase, and the ligation mixture transformed into *E. coli* as above. Plasmid DNA from several ampicillin resistant transformants were selected for analysis, and one plasmid DNA demonstrated the proper construction, and was designated pKCEAtrp207-I*.

E.1.8 Construction of a Plasmid Vector for Direct Expression of Mouse Mature Anti-CEA Heavy (Gamma 1) Chain Gene, pγCEAtrp207-1*

FIG. **7** illustrates the construction of pγCEAtrp207-1*. This plasmid was constructed in two parts beginning with construction of the C-terminal region of the gamma 1 gene.

5 μg of plasmid pHGH207-1* was digested with Ava I, extended to blunt ends with DNA polymerase I large fragment (Klenow fragment), extracted with phenol/$CHCl_3$, and ethanol precipitated. The DNA was digested with BamH I treated with BAP and the large fragment (fragment A) was purified by 6 percent polyacrylamide gel electrophoresis and electroelution.

~5 μg of pγ11 was digested with Pst I and the gamma chain cDNA insert fragment containing the C-terminal portion of the gene was purified, digested with Ava II followed by extension of the Ava II cohesive ends with Klenow, followed by Taq I digestion. The 375 basepair blunt ended Ava II to Taq I fragment (fragment B) was isolated and purified by gel electrophoresis and electroelution.

9 μg of pγ298 was digested with Taq I and BamH I for isolation of the 496 basepair fragment (fragment C).

Approximately equimolar amounts of fragments A, B, and C were ligated overnight at 14° in 20 μl reaction mixture, then transformed into *E. coli* strain 294. The plasmid DNA from six ampicillin resistant transformants was committed to restriction endonuclease analysis and one plasmid DNA, named pγCEAInt, demonstrated the correct construction of the C-terminal portion of gamma 1 (FIG. **5**).

To obtain the N-terminal sequences, 30 μg of pγ298 was digested with Pst I and the 628 basepair DNA fragment encoding the N-terminal region of mouse anti-CEA gamma chain was isolated and purified. This fragment was further digested with Alu I and Rsa I for isolation of the 280 basepair fragment. A 15 nucleotide DNA primer

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      met glu val met leu
5' ATG GAA GTG ATG CTG 3'
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was synthesized by the phosphotriester method (supra).

The 5' methionine serves as the initiation codon. 500 ng of this synthetic oligomer primer was phosphorylated at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl reaction mixture. ~500 ng of the 280 basepair Alu I-Rsa I DNA fragment was mixed with the phosphorylated primer. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with HpaII. ~50 ng of the expected 125 basepair blunt-end to Hpa II DNA fragment (fragment D) was purified from the gel.

A second aliquot of pγ298 DNA was digested with Pst I, the 628 basepair DNA fragment purified by polyacrylamide gel electrophoresis, and further digested with BamH I and Hpa II. The resulting 380 basepair fragment (fragment E) was purified by gel electrophoresis.

~5 μg of pγCEAIntI was digested with EcoR I, the cohesive ends were made flush with DNA polymerase I (Klenow), further digested with BamH I, treated with BAP and electrophoresed on a 6 percent polyacrylamide gel. The large vector fragment (fragment F) was isolated and purified.

In a three fragment ligation, 50 ng fragment D, 100 ng fragment E, and 100 ng fragment F were ligated overnight at 4° in a 20 μl reaction mixture and used to transform *E. coli* K12 strain 294. The plasmid DNAs from 12 ampicillin resistant transformants were analyzed for the correct construction and the nucleotide sequence surrounding the initiation codon was verified to be correct for the plasmid named pγCEAInt2.

The expression plasmid, pγCEAtrp207-I* used for expression of the heavy chain gene is prepared by a 3-way ligation using the large vector fragment from pBR322(XAP) (supra) and two fragments prepared from pγCEAInt2.

pBR322(XAP) was treated as above by digestion with EcoR1, blunt ending with DNA polymerase (Klenow) in the presence of dNTPs, followed by digestion with Pst I, and isolation of the large vector fragment by gel electrophoresis. A 1543 base pair fragment from pγCEAInt2 containing trp promoter linked with the N-terminal coding region of the heavy chain gene was isolated by treating pγCEAInt2 with Pst I followed by BamH I, and isolation of the desired fragment using PAGE. The 869 base pair fragment containing the C-terminal coding portion of the gene was prepared by partial digestion of pγCEAInt2 with Ava I, blunt ending with Klenow, and subsequent digestion with BamH I, followed by purification of the desired fragment by gel electrophoresis.

The aforementioned three fragments were then ligated under standard conditions using T4 DNA ligase, and a ligation mixture used to transform *E. coli* strain 294. Plasmid DNAs from several tetracycline resistant transformants were analyzed; one plasmid DNA demonstrated the proper construction and was designated pγCEAtrp207-1*.

E.1.9 Production of Immunoglobulin Chains by *E. coli*

E. coli strain W3110 (ATTC No. 27325) was transformed with pγCEAtrp207-1* or pKCEAtrp207-1* using standard techniques.

To obtain double transformants, *E. coli* strain W3110 cells were transformed with a modified pKCEAtrp207-1*, pKCEAtrp207-1*Δ, which had been modified by cleaving a Pst I-Pvu I fragment from the amp^R gene and religating. Cells transformed with pKCEAtrp207-1*Δ are thus sensitive to ampicillin but still resistant to tetracycline. Successful transformants were retransformed using pγCEAInt2 which confers resistance to ampicillin but not tetracycline. Cells containing both pKCEAtrp207-1*Δ and pγCEAInt2 thus identified by growth in a medium containing both ampicillin and tetracycline.

To confirm the production of heavy and/or light chains in the transformed cells, the cell samples were inoculated into M9 tryptophan free medium containing 10 μg/ml tetracycline, and induced with indoleacrylic acid (IAA) when the OD 550 reads 0.5. The induced cells were grown at 37° C. during various time periods and then spun down, and suspended in TE buffer containing 2 percent SDS and 0.1 M β-mercaptoethanol and boiled for 5 minutes. A 10×volume of acetone was added and the cells kept at 22° C. for 10 minutes, then centrifuged at 12,000 rpm. The precipitate was suspended in O'Farrell SDS sample buffer (O'Farrell, P. H., *J. Biol. Chem.,* 250: 4007 (1975)); boiled 3 minutes, recentrifuged, and fractionated using SDS PAGE (10 percent), and stained with silver stain (Goldman, D. et al., *Science* 211: 1437 (1981)); or subjected to Western blot using rabbit anti-mouse IgG (Burnett, W. N., et al., *Anal. Biochem.* 112: 195 (1981)), for identification light chain and heavy chain.

Cells transformed with pγCEAtrp207-1* showed bands upon SDS PAGE corresponding to heavy chain molecular weight as developed by silver stain. Cells transformed with pKCEAtrp207-1* showed the proper molecular weight band for light chain as identified by Western blot; double transformed cells showed bands for both heavy and light chain molecular weight proteins when developed using rabbit anti-mouse IgG by Western blot. These results are shown in FIGS. **8**A, **8**B, and **8**C.

FIG. **8**A shows results developed by silver stain from cells transformed with pγCEAtrp207-1*. Lane 1 is monoclonal anti-CEA heavy chain (standard) from CEA.66-E3. Lanes 2b–5b are timed samples 2 hrs, 4 hrs, 6 hrs, and 24 hrs after IAA addition. Lanes 2a–5a are corresponding untransformed controls; Lanes 2c–5c are corresponding uninduced transformants.

FIG. **8**B shows results developed by Western blot from cells transformed with pKCEAtrp207-1*. Lanes 1b–6b are extracts from induced cell immediately, 1 hr, 3.5 hrs, 5 hrs, 8 hrs, and 24 hrs after IAA addition, and 1a–6a corresponding uninduced controls. Lane 7 is an extract from a pγCEAtrp207-1* control, lanes 8, 9, and 10 are varying amounts of anti CEA-kappa chain from CEA.66-E3 cells.

FIG. **8**C shows results developed by Western blot from four colonies of double transformed cells 24 hours after IAA addition (lanes 4–7). Lanes 1–3 are varying amounts of monoclonal gamma chain controls, lanes 8 and 9 are untransformed and pγCEAtrp207-1* transformed cell extracts, respectively.

In another quantitative assay, frozen, transformed *E. coli* cells grown according to E.1.10 (below) were lysed by heating in sodium dodecyl sulfate (SDS)/β-mercaptoethanol cell lysis buffer at 100°. Aliquots were loaded on an SDS polyacrylamide gel next to lanes loaded with various amounts of hybridoma anti-CEA. The gel was developed by the Western blot, Burnett (supra), using ^{125}I-labeled sheep anti-mouse IgG antibody from New England Nuclear. The results are shown in FIG. **9**. The figure shows that the *E. coli* products co-migrate with the authentic hybridoma chains, indicating no detectable proteolytic degradation in *E. coli*. Heavy chain from mammalian cells is expected to be slightly heavier than *E. coli* material due to glycosylation in the former. Using the hybridoma lanes as a standard, the following estimates of heavy and light chain production were made:

	(Per gram of cells)
E. coli (W3110/pγCEAtrp207-1*)	5 mg γ
E. coli (W3110/pKCEAtrp207-1*)	1.5 mg K
E. coli (W3110/pKCEAtrp207-1*A, pγCEAInt2)	0.5 mg K, 1.0 mg γ

E.1.10 Reconstitution of Antibody from Recombinant K and Gamma Chains

In order to obtain heavy and light chain preparations for reconstitution, transformed cells were grown in larger batches, harvested and frozen. Conditions of growth of the variously transformed cells were as follows:

E. coli (W3110/pγCEAtrp207-1*) were inoculated into 500 ml LB medium containing 5 μg/ml tetracycline and grown on a rotary shaker for 8 hours. The culture was then transferred to 10 liters of fermentation medium containing yeast nutrients, salts, glucose, and 2 μg/ml tetracycline. Additional glucose was added during growth and at OD 550=20, indoleacrylic (IAA), a trp derepressor, was added to a concentration of 50 μg/ml. The cells were fed additional glucose to a final OD 550=40, achieved approximately 6 hours from the IAA addition.

E. coli (W3110) cells transformed with pKCEA trp 207-1* and double transformed (with pKCEAtrp207-1*Δ and pγCEAInt2) were grown in a manner analogous to that described above except that the OD 550 six hours after IAA addition at harvest was 25–30.

The cells were then harvested by centrifugation, and frozen.

E.2 Assay Method for Reconstituted Antibody

Anti-CEA activity was determined by ELISA as a criterion for successful reconstitution. Wells of microtiter plates (Dynatech Immulon) were saturated with CEA by incubating 100 μl of 2–5 μg CEA/ml solution in 0.1M carbonate buffer, pH 9.3 for 12 hours at room temperature. The wells were then washed 4 times with phosphate buffered saline (PBS), and then saturated with BSA by incubating 200 μl of 0.5 percent BSA in PBS for 2 hours at 37° C., followed by washing 4 times with PBS. Fifty microliters of each sample was applied to each well. A standard curve (shown in FIG. **10**), was run, which consisted of 50 μl samples of 10 μg, 5 μg, 1 μg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng and 1 ng anti-CEA/ml in 0.5 percent BSA in PBS, plus 50 μl of 0.5 percent BSA in PBS alone as a blank. All of the samples were incubated in the plate for 90 minutes at 37° C.

The plates were then washed 4 times with PBS, and sheep anti-mouse IgG-alkaline phosphate (TAGO, Inc.) was applied to each well by adding 100 μl of an enzyme concentration of 24 units/ml in 0.5 percent BSA in PBS. The solution was incubated at 37° C. for 90 minutes. The plates were washed 4 times with PBS before adding the substrate, 100 μl of a 0.4 mg/ml solution of p-nitrophenylphosphate (Sigma) in ethanolamine buffered saline, pH 9.5. The substrate was incubated 90 minutes at 37° C. for color development.

The A_{450} of each well was read by the Microelisa Auto Reader (Dynatech) set to a threshold of 1.5, calibration of 1.0 and the 0.5 percent BSA in PBS (Blank) well set to 0.000. The A_{450} data was tabulated in RS-1 on the VAX system, and the standard curve data fitted to a four-parameter logistic model. The unknown samples' concentration were calculated based on the A_{450} data.

E.3 Reconstitution of Recombinant Antibody and Assay

Frozen cells prepared as described in paragraph E.1.10 were thawed in cold lysis buffer [10 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication. The lysate was partially clarified by centrifugation for 20 mins at 30,000 rpm. The supernatant was protected from proteolytic enzymes by an additional 1 mM PMSF, and used immediately or stored frozen at −80° C.; frozen lysates were never thawed more than once.

The S-sulfonate of *E. coli* produced anti-CEA heavy chain (γ) was prepared as follows: Recombinant *E. coli* cells transformed with pγCEAtrp207-1* which contained heavy chain as insoluble bodies, were lysed and centrifuged as above; the pellet was resuspended in the same buffer, sonicated and re-centrifuged. This pellet was washed once with buffer, then suspended in 6M guanidine HCl, 0.1M Tris HCl, pH 8, 1 mM EDTA, 20 mg/ml sodium sulfite and 10 mg/ml sodium tetrathionate and allowed to react at 25° for about 16 hrs. The reaction mixture was dialyzed against 8M urea, 0.1M Tris HCl, pH 8, and stored at 4°, to give a 3 mg/ml solution of γ-$SS0_3$.

650 μl of cell lysate from cells of various *E. coli* strains producing various IgG chains, was added to 500 mg urea. To this was added β-mercaptoethanol to 20 mM, Tris-HCl, pH 8.5 to 50 mM and EDTA to 1 mM, and in some experiments, γ-$SS0_3$ was added to 0.1 mg/ml. After standing at 25° for 30–90 mins., the reaction mixtures were dialyzed at 4° against a buffer composed of 0.1M sodium glycinate, pH 10.8, 0.5M urea, 10 mM glycine ethyl ester, 5 mM reduced glutathione, 0.1 mM oxidized glutathione. This buffer was prepared from N_2-saturated water and the dialysis was performed in a capped Wheaton bottle. After 16–48 hours, dialysis bags were transferred to 4° phosphate buffered saline containing 1 mM PMSF and dialysis continued another 16–24 hrs. Dialysates were assayed by ELISA as described in paragraph E.2 for ability to bind CEA. The results below show the values obtained by comparison with the standard curve in x ng/ml anti-CEA. Also shown are the reconstitution efficiencies calculated from the ELISA responses, minus the background (108 ng/ml) of cells producing K chain only, and from estimates of the levels of γ and K chains in the reaction mixtures.

	ng/ml anti-CEA	Percent recombination
E. coli W3110 producing IFN-αA (control)	0	—
E. coli (W3110/pKCEAtrp207-1*)	108	—
E. coli (W3110/pKCEAtrp207-1*), plus γ-SSO_3	848	0.33
E. coli (W3110/pKCEAtrp207-1*Δ, pγCEAInt2)	1580	0.76
Hybridoma anti-CEA K-SSO_3 and γ-SSO_3	540	0.40

E.4 Preparation of Chimeric Antibody

FIGS. **11** and **12** show the construction of an expression vector for a chimeric heavy (gamma) chain which comprises the murine anti CEA variable region and human γ-2 constant region.

A DNA sequence encoding the human gamma-2 heavy chain is prepared as follows: the cDNA library obtained by standard techniques from a human multiple myeloma cell line is probed with 5' GGGCACTCGACACAA 3 ' to obtain the plasmid containing the cDNA insert for human gamma-2 chain (Takahashi, et al., *Cell,* 29: 671 (1982), incorporated herein by reference), and analyzed to verify its identity with the known sequence in human gamma-2 (Ellison, J., et al., *Proc. Natl. Acad. Sci.* (*USA*), 79: 1984 (1982) incorporated herein by reference).

As shown in FIG. **11**, two fragments are obtained from this cloned human gamma 2 plasmid (pγ2). The first fragment is formed by digestion with PvuII followed by digestion with Ava III, and purification of the smaller DNA fragment, which contains a portion of the constant region, using 6 percent PAGE. The second fragment is obtained by digesting the pγ2 with any restriction enzyme which cleaves in the 3' untranslated region of γ2, as deduced from the nucleotide sequence, filling in the Klenow and dNTPs, cleaving with Ava III, and isolating the smaller fragment using 6 percent PAGE. (The choice of a two step, two fragment composition to supply the PvuII-3' untranslated fragment provides a cleaner path to product due to the proximity of the AvaIII site to the 3 terminal end thus avoiding additional restriction sites in the gene sequence matching the 3' untranslated region site.) pγCEA207-1* is digested with EcoR 1, treated with Klenow and dNTPs to fill in the cohesive end, and digested with Pvu II, the large vector fragment containing promoter isolated by 6 percent PAGE.

The location and DNA sequence surrounding the PvuII site in the mouse gamma-1 gene are identical to the location and DNA sequence surrounding the PvuII site in the human gamma-2 gene.

The plasmid resulting from a three way ligation of the foregoing fragments, pChim1, contains, under the influence of trp promoter, the variable and part of the constant region of murine anti-CEA gamma 1 chain, and a portion of the gamma 2 human chain. pChim1 will, in fact, express a chimeric heavy chain when transformed into *E. coli,* but one wherein the change from mouse to human does not take place at the variable to constant junction.

FIG. **12** shows modification of pChim1 to construct pChim2 so that the resulting protein from expression will contain variable region from murine anti CEA antibody and constant region from the human γ-2 chain. First, a fragment is prepared from pChim1 by treating with Nco I, blunt ending with Klenow and dNTPs, cleaving with Pvu II, and isolating the large vector fragment which is almost the complete plasmid except for short segment in the constant coding region for mouse anti CEA. A second fragment is prepared from the previously described pγ2 by treating with Pvu II, followed by treating with any restriction enzyme which cleaves in the variable region, blunt ending with Klenow and dNTPs and isolating the short fragment which comprises the junction between variable and constant regions of this chain.

Ligation of the foregoing two fragments produces an intermediate plasmid which is correct except for an extraneous DNA fragment which contains a small portion of the constant region of the murine anti CEA antigen, and a small portion of the variable region of the human gamma chain. This repair can be made by excising the Xba I to Pvu II fragment and cloning into M13 phage as described by Messing et al., *Nucleic Acids Res.* 9: 309 (1981), followed by in vitro site directed deletion mutagenesis as described by Adelman, et al., *DNA,* in press (1983) which is incorporated

herein by reference. The Xba I-Pvu II fragment thus modified is ligated back into the intermediate plasmid to form pChim2. This plasmid then is capable of expressing in a suitable host a cleanly constructed murine variable/human constant chimeric heavy chain.

In an analogous fashion, but using mRNA templates for cDNA construction for human kappa rather than γ chain, the expression plasmid for chimeric light chain is prepared.

The foregoing two plasmids are then double transformed into *E. coli* W3110, the cells grown and the chains reconstituted as set forth in paragraph E.1–E.3 supra.

E.5 Preparation of Altered Murine Anti-CEA Antibody

E.5.1 Construction of Plasmid Vectors for Direct Expression of Altered Murine Anti-CEA Heavy Chain Gene

The cysteine residues, and the resultant disulfide bonds in the region of amino acids 216–230 in the constant region of murine anti-CEA heavy chain are suspected to be important for complement fixation (Klein, et al., *Proc. Natl. Acad. Sci., (USA)*, 78: 524 (1981)) but not for the antigen binding property of the resulting antibody. To decrease the probability of incorrect disulfide bond formation during reconstitution according to the process of the invention herein, the nucleotides encoding the amino acid residues 226–232 which includes codons for three cysteines, are deleted as follows:

A "deleter" deoxyoligonucelotide, 5' CTAACACCATGTCAGGGT is used to delete the relevant portions of the gene from pγCEAtrp207-1* by the procedure of Wallace, et al., *Science,* 209: 1396 (1980) or of Adelman, et al., *DNA* 2, 183 (1983). Briefly, the "deleter" deoxyoligonucelotide is annealed with denatured pγCEAtrp207-1* DNA, and primer repair synthesis carried out in vitro, followed by screening by hybridization of presumptive deletion clones with P^{32} labelled deleter sequence.

E.5.2 Production of Cysteine Deficient Altered Antibody

The plasmid prepared in E.5.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains, and the altered antibody reconstituted as described in E.1.10.

E.6 Preparation of Fab

E.6.1 Construction of a Plasmid Vector for Direct Expression of Murine Anti-CEA Gamma 1 Fab Fragment Gene pγCEAFabtrp207-1*

FIG. **13** presents the construction of pγCEAFabtrp207-1*. 5 μg of pBR322 was digested with Hind III, the cohesive ends made flush by treating with Klenow and dNTPs; digested with Pst I, and treated with BAP. The large vector fragment, fragment I, was recovered using 6 percent PAGE followed by electroelution.

5 μg of pγCEAtrp207-1* was digested with both BamH I and Pst I and the ~1570 bp DNA fragment (fragment II) containing the trp promoter and the gene sequence encoding the variable region continuing into constant region and further into the anti-CEA gamma 1 chain hinge region, was isolated and purified after electrophoresis.

Expression of the anti-CEA gamma 1 chain Fab fragment rather than complete heavy chain requires that a termination codon be constructed at the appropriate location in the gene. For this, the 260 bp Nco I-Nde I DNA fragment from 20 μg of the pγ298 was isolated and purified. A 13 nucleotide DNA primer, the complement of which encodes the last 3 C-terminal amino acids of the Fab gene and 2 bases of the 3 needed for the stop codon, was synthesized by the phosphotriester method (supra). The probe hybridizes to nucleotides 754 to 767 (FIG. **4**) which has the following sequence:

```
AspCysGlyStop
5' GGGATTGTGGTTG  3'
```

The third base of the stop codon is provided by the terminal nucleotide of the filled-in Hind III site from pBR322 cleavage described above. 500 ng of this primer was used in a primer repair reaction by phosphorylation at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl, and mixing with ~200 ng of the Nco I-Nde I DNA fragment. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, digested with BamH I and the reaction electrophoresed through a 6 percent polyacrylamide gel. ~50 ng of the 181 bp blunt end to BamH I DNA fragment, fragment III, was isolated and purified.

~100 ng of fragment I, ~100 ng each of fragments II and III were ligated overnight and transformed into *E. coli* K12 strain 294. Plasmid DNA from several tetracycline resistant transformants was analyzed for the proper construction and the nucleotide sequence through the repair blunt end filled-in Hind III junction was determined for verification of the TGA stop codon.

E.6.2 Production of Fab Protein

The plasmid prepared in E.6.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains and the Fab protein reconstituted as described in E.1.10.

What is claimed is:

1. A process for producing an immunoglobulin molecule or an immunologically functional immunoglobulin fragment comprising at least the variable domains of the immunoglobulin heavy and light chains, in a single host cell, comprising the steps of:

(i) transforming said single host cell with a first DNA sequence encoding at least the variable domain of the immunoglobulin heavy chain and a second DNA sequence encoding at least the variable domain of the immunoglobulin light chain, and

(ii) independently expressing said first DNA sequence and said second DNA sequence so that said immunoglobulin heavy and light chains are produced as separate molecules in said transformed single host cell.

2. The process according to claim **1** wherein said first and second DNA sequences are present in different vectors.

3. The process according to claim **1** wherein said first and second DNA sequences are present in a single vector.

4. A process according to claim **3** wherein the vector is a plasmid.

5. The process according to claim **4** wherein the plasmid is pBR322.

6. The process according to claim **1** wherein the host cell is a bacterium or yeast.

7. The process according to claim **6** wherein the host cell is *E. coli* or *S. cerevisiae.*

8. A process according to claim **7** wherein the host cell is *E. coli* strain X1776 (ATCC No. 31537).

9. A process according to claim **1** wherein the immunoglobulin heavy and light chains are expressed in the host cell and secreted therefrom as an immunologically functional immunoglobulin molecule or immunoglobulin fragment.

10. A process according to claim **1** wherein the immunoglobulin heavy and light chains are produced in insoluble form and are solubilized and allowed to refold in solution to form an immunologically functional immunoglobulin molecule or immunoglobulin fragment.

11. A process according to claim **1** wherein the DNA sequences code for the complete immunoglobulin heavy and light chains.

12. The process according to claim **1** wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from the same source as the variable domain to which it is attached.

13. The process according to claim **1** wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from a species or class different from that from which the variable domain to which it is attached is derived.

14. The process according to claim **1** wherein said first and second DNA sequences are derived from one or more monoclonal antibody producing hybridomas.

15. A vector comprising a first DNA sequence encoding at least a variable domain of an immunoglobulin heavy chain and a second DNA sequence encoding at least a variable domain of an immunoglobulin light chain wherein said first DNA sequence and said second DNA sequence are located in said vector at different insertion sites.

16. A vector according to claim **15** which is a plasmid.

17. A host cell transformed with a vector according to claim **15**.

18. A transformed host cell comprising at least two vectors, at least one of said vectors comprising a DNA sequence encoding at least a variable domain of an immunoglobulin heavy chain and at least another one of said vectors comprising a DNA sequence encoding at least the variable domain of an immunoglobulin light chain.

19. The process of claim **1** wherein the host cell is a mammalian cell.

20. The transformed host cell of claim **18** wherein the host cell is a mammalian cell.

21. A method comprising

a) preparing a DNA sequence consisting essentially of DNA encoding an immunoglobulin consisting of an immunoglobulin heavy chain and light chain or Fab region, said immunoglobulin having specificity for a particular known antigen;

b) inserting the DNA sequence of step a) into a replicable expression vector operably linked to a suitable promoter;

c) transforming a prokaryotic or eukaryotic microbial host cell culture with the vector of step b);

d) culturing the host cell; and

e) recovering the immunoglobulin from the host cell culture, said immunoglobulin being capable of binding to a known antigen.

22. The method of claim **21** wherein the heavy and light chain are the heavy and light chains of anti-CEA antibody.

23. The method of claim **21** wherein the heavy chain is of the gamma family.

24. The method of claim **21** wherein the light chain is of the kappa family.

25. The method of claim **21** wherein the vector contains DNA encoding both a heavy chain and a light chain.

26. The method of claim **21** wherein the host cell is *E. coli* or yeast.

27. The method of claim **26** wherein the heavy chain and light chains or Fab region are deposited within the cells as insoluble particles.

28. The method of claim **27** wherein the heavy and light chains are recovered from the particles by cell lysis followed by solubilization in denaturant.

29. The method of claim **21** wherein the heavy and light chains are secreted into the medium.

30. The method of claim **21** wherein the host cell is a gram negative bacterium and the heavy and light chains are secreted into the periplasmic space of the host cell bacterium.

31. The method of claim **21** further comprising recovering both heavy and light chain and reconstituting light chain and heavy chain to form an immunoglobulin having specific affinity for a particular known antigen.

32. The insoluble particles of heavy chain and light chains or Fab region produced by the method of claim **27**.

33. A process for producing an immunoglobulin molecule or an immunologically functional immunoglobulin fragment comprising at least the variable domains of the immunoglobulin heavy and light chains, in a single host cell, comprising:

independently expressing a first DNA sequence encoding at least the variable domain of the immunoglobulin heavy chain and a second DNA sequence encoding at least the variable domain of the immunoglobulin light chain so that said immunoglobulin heavy and light chains are produced as separate molecules in said single host cell transformed with said first and second DNA sequences.

34. The process of claim **9**, further comprising the step of attaching the immunoglobulin molecule or immunoglobulin fragment to a label or drug.

35. The process of claim **10**, further comprising the step of attaching the immunoglobulin molecule or immunoglobulin fragment to a label or drug.

36. The process of claim **33**, further comprising the step of attaching the immunoglobulin molecule or immunoglobulin fragment to a label or drug.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,331,415 B1
DATED : December 18, 2001
INVENTOR(S) : Shmuel Cabilly et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [73], Assignee, please insert -- City of Hope, Duarte, CA (US) --.

Column 19,
Line 56, please change "BamH 1" to -- BamH I --.

Signed and Sealed this

Twenty-fifth Day of June, 2002

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

Attest:

Attesting Officer

US006331415C1

(12) EX PARTE REEXAMINATION CERTIFICATE (6829th)

United States Patent

Cabilly et al.

(10) Number: US 6,331,415 C1

(45) Certificate Issued: May 19, 2009

(54) **METHODS OF PRODUCING IMMUNOGLOBULINS, VECTORS AND TRANSFORMED HOST CELLS FOR USE THEREIN**

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(51) **Int. Cl.**

C12N 15/13	(2006.01)
C12N 15/00	(2006.01)
C12N 15/63	(2006.01)

(52) **U.S. Cl.** **435/69.6**; 435/252.1; 435/252.3; 435/252.33; 435/254.11; 435/254.2; 435/254.21; 435/69.7; 435/70.21; 435/71.2; 435/71.1; 435/70.1; 435/320.1; 435/455; 435/483; 435/485; 435/471; 435/69.1

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

The invention relates to processes for producing an immunoglobulin or an immunologically functional immunoglobulin fragment containing at least the variable domains of the immunoglobulin heavy and light chains. The processes can use one or more vectors which produce both the heavy and light chains or fragments thereof in a single cell. The invention also relates to the vectors used to produce the immunoglobulin or fragment, and to cells transformed with the vectors.

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Deposition transcript of Herbert Heyneker (Oct. 28, 1997), with exhibits.
Deposition transcript of William E. Holmes (Oct. 19, 2007), with exhibits.
Deposition transcript of Arthur D. Riggs, M.D. (Nov. 16, 2007).
Deposition transcript of Ronald Burnell Wetzel (Nov. 29, 2007), with exhibits.
Deposition transcript of Ian M. Armitage (Oct. 16, 2007), with exhibits.
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EX PARTE REEXAMINATION CERTIFICATE ISSUED UNDER 35 U.S.C. 307

THE PATENT IS HEREBY AMENDED AS INDICATED BELOW.

Matter enclosed in heavy brackets [ ] appeared in the patent, but has been deleted and is no longer a part of the patent; matter printed in italics indicates additions made to the patent.

AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

The patentability of claims **1**–**20** and **33**–**36** is confirmed.

Claims **21**, **27** and **32** are determined to be patentable as amended.

Claims **22**–**26** and **28**–**31**, dependent on an amended claim, are determined to be patentable.

21. A method comprising

a) preparing a *first* DNA sequence **[**consisting essentially of DNA**]** encoding an immunoglobulin **[**consisting of

an immunoglobulin**]** heavy chain and *a second DNA sequence encoding an immunoglobulin* light chain **[**or Fab region, said immunoglobulin having specificity for a particular known antigen**]**;

b) inserting the DNA **[**sequence**]** *sequences* of step a) into a replicable expression vector *wherein each sequence is* operably linked to a suitable promoter;

c) transforming a prokaryotic or eukaryotic microbial host cell culture with the vector of step b);

d) culturing the host cell *so that said immunoglobulin heavy and light chains are produced as separate molecules in said transformed host cell*; and

e) recovering the immunoglobulin from the host cell culture, said immunoglobulin being capable of binding to a known antigen.

27. The method of claim **26** wherein the heavy chain and light **[**chains or Fab region**]** *chain* are deposited within the cells as insoluble particles.

32. The insoluble particles of heavy chain and light chains **[**or Fab region**]** produced by the method of claim **27**.

* * * * *

EXHIBIT B

US007923221B1

(12) **United States Patent**
Cabilly et al.

(10) **Patent No.: US 7,923,221 B1**
(45) **Date of Patent: *Apr. 12, 2011**

(54) **METHODS OF MAKING ANTIBODY HEAVY AND LIGHT CHAINS HAVING SPECIFICITY FOR A DESIRED ANTIGEN**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **08/422,187**

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(58) **Field of Classification Search** 435/69.6, 435/69.1, 325, 252.3; 530/387.3
See application file for complete search history.

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(57) **ABSTRACT**

The invention relates to processes for producing an immunoglobulin or an immunologically functional immunoglobulin fragment containing at least the variable domains of the immunoglobulin heavy and light chains. The processes can use one or more vectors which produce both the heavy and light chains or fragments thereof in a single cell. The invention also relates to the vectors used to produce the immunoglobulin or fragment, and to cells transformed with the vectors.

47 Claims, 19 Drawing Sheets

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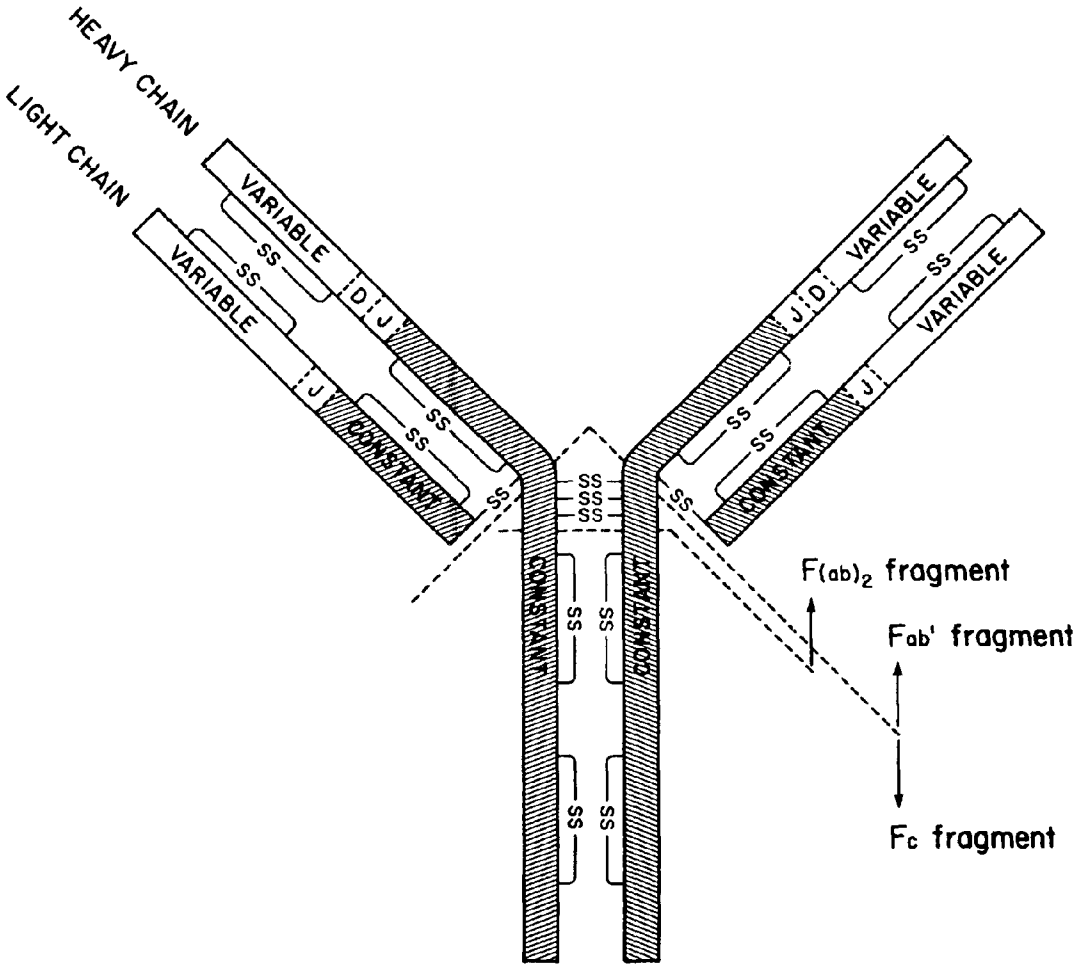
HEAVY CHAIN
LIGHT CHAIN
VARIABLE
CONSTANT
D
J
SS
F(ab)2 fragment
Fab' fragment
Fc fragment

Fig 1

tth111
hphI
haeIII
haeI
GTTGCTGTGG TTGTCTGGTG TTGAAGGAGA CATTGTGATG ACCCAGTCTC ACAAATTCAT GTCCACATCA GTAGGAGACA GGGTCAGCAT CACCTGCAAG
CAACGACACC AACAGACCAC AACTTCCTCT GTAACACTAC TGGGTCAGAG TGTTTAAGTA CAGGTGTAGT CATCCTCTGT CCCAGTCGTA GTGGACGTTC
sfaNI
scrFI
nciI
hpaII
hinfI
fnu4HI
bbv
scrFI
ecoRII
fokI
GCCAGTCAGG ATGTGGGTGC TGCTATAGCC TGGTATCAAC AGAAACCAGG ACAATCTCCT AAACTACTGA TTTACTGGGC ATCCACCCGG CACACTGGAG
CGGTCAGTCC TACACCCACG ACGATATCGG ACCATAGTTG TCTTTGGTCC TGTTAGAGGA TTTGATGACT AAATGACCCG TAGGTGGGCC GTGTGACCTC
fokI
sfaNI
xhoII
sau3A
dpnI
hphI
hincII
sau3A
dpnI
TCCCTGATCG CTTCACAGGC AGTGGATCTG GGACAGATTT CACTCTCACC ATTAGCAATG TGCAGTCTGA TGACTTGGCA GATTATTTCT GTCAACAATA
AGGGACTAGC GAAGTGTCCG TCACCTAGAC CCTGTCTAAA GTGAGAGTGG TAATCGTTAC ACGTCAGACT ACTGAACCGT CTAATAAAGA CAGTTGTTAT
fnu4HI
bbv
hpaI
hincII
sau96
aval1
aluI
aluI
sfaNI
mboII
TAGCGGGTAT CCTCTCACGT TCGGTGCTGG GACCAAGCTG GAGCTGAAAC GGGCTGATGC TGCACCAACT GTATCCATCT TCCCACCATC CAGTGAGCAG
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fokI
mnlI
ddeI
mnlI
xmnI
mboII
acyI
TTAACATCTG GAGGTGCCTC AGTCGTGTGC TTCTTGAACA ACTTCTACCC CAAAGACATC AATGTCAAGT GGAAGATTGA TGGCAGTGAA CGACAAAATG
AATTGTAGAC CTCCACGGAG TCAGCACACG AAGAACTTGT TGAAGATGGG GTTTCTGTAG TTACAGTTCA CCTTCTAACT ACCGTCACTT GCTGTTTTAC

Fig. 2A.

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                                  sau3A
                                  dpnI                                                 fnu4HI
           hgaI                 bclI                                                   bbv        mnlI   hincII                                            aluI
501        GCGTCCTGAA CAGTTGGACT GATCAGGACA GCAAAGACAG CACCTACAGC ATGAGCAGCA CCCTCACGTT GACCAAGGAC GAGTATGAAC GACATAACAG
           CGCAGGACTT GTCAACCTGA CTAGTCCTGT CGTTTCTGTC GTGGATGTCG TACTCGTCGT GGGAGTGCAA CTGGTTCCTG CTCATACTTG CTGTATTGTC

                      mnlI                                                                                       sau96    hgaI
                        haeIII                                                                                       ddeI
                       haeI                   hphI              aluI                                             avaII    acyI
601        CTATACCTGT GAGGCCACTC ACAAGACATC AACTTCACCC ATTGTCAAGA GCTTCAACAG GAATGAGTGT TAGAGACAAA GGTCCTGAGA CGCCACCACC
           GATATGGACA CTCCGGTGAG TGTTCTGTAG TTGAAGTGGG TAACAGTTCT CGAAGTTGTC CTTACTCACA ATCTCTGTTT CCAGGACTCT GCGGTGGTGG

                                                                                                                         mnlI
           aluI      aluI               mboII     ddeI           mnlI                                 hgiA          mnlI           mnlI
701        AGCTCCCCAG CTCCATCCTA TCTTCCCTTC TAAGGTCTTG GAGGCTTCCC CACAAGCGAC CTACCACTGT TGCGGTGCTC CAAACCTCCT CCCCACCTCC
           TCGAGGGGTC GAGGTAGGAT AGAAGGGAAG ATTCCAGAAC CTCCGAAGGG GTGTTCGCTG GATGGTGACA ACGCCACGAG GTTTGGAGGA GGGGTGGAGG
                fokI

                  mnlI
               mnlI   mnlI                                      xmnI                   hinfI
801        TTCTCCTCCT CCTCCCTTTC CTTGGCTTTT ATCATGCTAA TATTTGCAGA AAATATTCAA TAAAGTGAGT CTTTGCACTT GA
           AAGAGGAGGA GGAGGGAAAG GAACCGAAAA TAGTACGATT ATAAACGTCT TTTATAAGTT ATTTCACTCA GAAACGTGAA CT

nucleotides: 882
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Fig. 2B.

```
    -9                                  1                                   10                                      20
    leu leu trp leu ser gly val glu gly asp ile val met thr gln ser his lys phe met ser thr ser val gly asp arg val ser
G   UUG CUG UGG UUG UCU GGU GUU GAA GGA GAC AUU GUG AUG ACC CAG UCU CAC AAA UUC AUG UCC ACA UCA GUA GGA GAC AGG GUC AGC

                                    30                                      40                                      50
ile thr cys lys ala ser gln asp val gly ala ala ile ala trp tyr gln gln lys pro gly gln ser pro lys leu leu ile tyr trp
AUC ACC UGC AAG GCC AGU CAG GAU GUG GGU GCU GCU AUA GCC UGG UAU CAA CAG AAA CCA GGA CAA UCU CCU AAA CUA CUG AUU UAC UGG

                                    60                                      70                                      80
ala ser thr arg his thr gly val pro asp arg phe thr gly ser gly ser gly thr asp phe thr leu thr ile ser asn val gln ser
GCA UCC ACC CGG CAC ACU GGA GUC CCU GAU CGC UUC ACA GGC AGU GGA UCU GGG ACA GAU UUC ACU CUC ACC AUU AGC AAU GUG CAG UCU

                                    90                                      100                                     110
asp asp leu ala asp tyr phe cys gln gln tyr ser gly tyr pro leu thr phe gly ala gly thr lys leu glu leu lys arg ala asp
GAU GAC UUG GCA GAU UAU UUC UGU CAA CAA UAU AGC GGG UAU CCU CUC ACG UUC GGU GCU GGG ACC AAG CUG GAG CUG AAA CGG GCU GAU

                                    120                                     130                                     140
ala ala pro thr val ser ile phe pro pro ser ser glu gln leu thr ser gly gly ala ser val val cys phe leu asn asn phe tyr
GCU GCA CCA ACU GUA UCC AUC UUC CCA CCA UCC AGU GAG CAG UUA ACA UCU GGA GGU GCC UCA GUC GUG UGC UUC UUG AAC AAC UUC UAC

                                    150                                     160                                     170
pro lys asp ile asn val lys trp lys ile asp gly ser glu arg gln asn gly val leu asn ser trp thr asp gln asp ser lys asp
CCC AAA GAC AUC AAU GUC AAG UGG AAG AUU GAU GGC AGU GAA CGA CAA AAU GGC GUC CUG AAC AGU UGG ACU GAU CAG GAC AGC AAA GAC

                                    180                                     190                                     200
ser thr tyr ser met ser ser thr leu thr leu thr lys asp glu tyr glu arg his asn ser tyr thr cys glu ala thr his lys thr
AGC ACC UAC AGC AUG AGC AGC ACC CUC ACG UUG ACC AAG GAC GAG UAU GAA CGA CAU AAC AGC UAU ACC UGU GAG GCC ACU CAC AAG ACA

                                    210             214
ser thr ser pro ile val lys ser phe asn arg asn glu cys AM
UCA ACU UCA CCC AUU GUC AAG AGC UUC AAC AGG AAU GAG UGU UAG AGACAAAGGUCCUGAGACGCCACCACCAGCUCCCCAGCUCCAUCCUAUCUUCCCUUCUAA

GGUCUUGGAGGCUUCCCCACAAGCGACCUACCACUGUUGCGGUGCUCCAAACCUCCUCCCCACCUCCUUCUCCUCCUCCUCCCUUUCCUUGGCUUUUAUCAUGCUAAUAUUUGCAGAAAA

UAUUCAAUAAAGUGAGUCUUUGCACUUGA
```

Fig. 3

```
                        sau96                           ddeI
      hinfI             avaII mnlI                         aluI                               ahaIII          sfaNI
1     GAGTCAGCAC TGAACACGGA CCCCTCACGA TGAACTTCGG GCTCAGCTTG ATTTACCTTG TCCTTGTTTT AAAAGTTGTC CAGTGTGAAG TGATGCTGGT
      CTCAGTCGTG ACTTGTGCCT GGGGAGTGCT ACTTGAAGCC CGAGTCGAAC TAAATGGAAC AGGAACAAAA TTTTCAACAG GTCACACTTC ACTACGACCA

                                   scrFI    sau96
                                       mnlI                          fnu4HI
        hinfI      hinfI           ecoRII   avaII                    bbv mnlI    hinfI
101   GGAGTCTGGG GGAGTCTTAA TGGAGCCTGG AGGGTCCCTG AAACTCTCCT GTGCAGCCTC TGGATTCACT TTCAGTAGAT ATGCCATGTC TTGGGTTCGC
      CCTCAGACCC CCTCAGAATT ACCTCGGACC TCCCAGGGAC TTTGAGAGGA CACGTCGGAG ACCTAAGTGA AAGTCATCTA TACGGTACAG AACCCAAGCG

           hpaII     mnlI                                                                                 hphI
         hinfI      mboII                                                                              hinfI
201   CAGACTCCGG AGAAGAGGCT GGAGTGGGTC GCAACCATTA GTAGTGGTGG TAGTTCACAC CTTCCATCCA GACAGTGTGA AGGGCGATTC ACCATCTCCA
      GTCTGAGGCC TCTTCTCCGA CCTCACCCAG CGTTGGTAAT CATCACCACC ATCAAGTGTG GAAGGTAGGT CTGTCACACT TCCCGCTAAG TGGTAGAGGT
                                                                 fokI

                                                          mnlI     mnlI
                                  rsaI                  ddeI    ddeI        haeIII                 mnlI
301   GAGACAATGC CAAGAACACC CTGTACCTGC AAATGAGCAG TCTGAGGTCT GAGGACACGG CCATGTATTA CTGTGCAAGA CCCCCTCTTA TTTCGTTAGT
      CTCTGTTACG GTTCTTGTGG GACATGGACG TTTACTCGTC AGACTCCAGA CTCCTGTGCC GGTACATAAT GACACGTTCT GGGGGAGAAT AAAGCAATCA

                                                                                                               xhoII
                                                                                                            scrFI
                                                                                                       sau96    sau3A
                                                        mnlI                mnlI                            ecoRII
                                                         ddeI   hphI         ddeI                      haeIII    dpnI
401   AGCGGACTAT GCTATGGACT ACTGGGGTCA AGGAACCTCA GTCACCGTCT CCTCAGCCAA AACGACACCC CCATCTGTCT ATCCACTGGC CCCTGGATCT
      TCGCCTGATA CGATACCTGA TGACCCCAGT TCCTTGGAGT CAGTGGCAGA GGAGTCGGTT TTGCTGTGGG GGTAGACAGA TAGGTGACCG GGGACCTAGA
```

Fig. 4A.

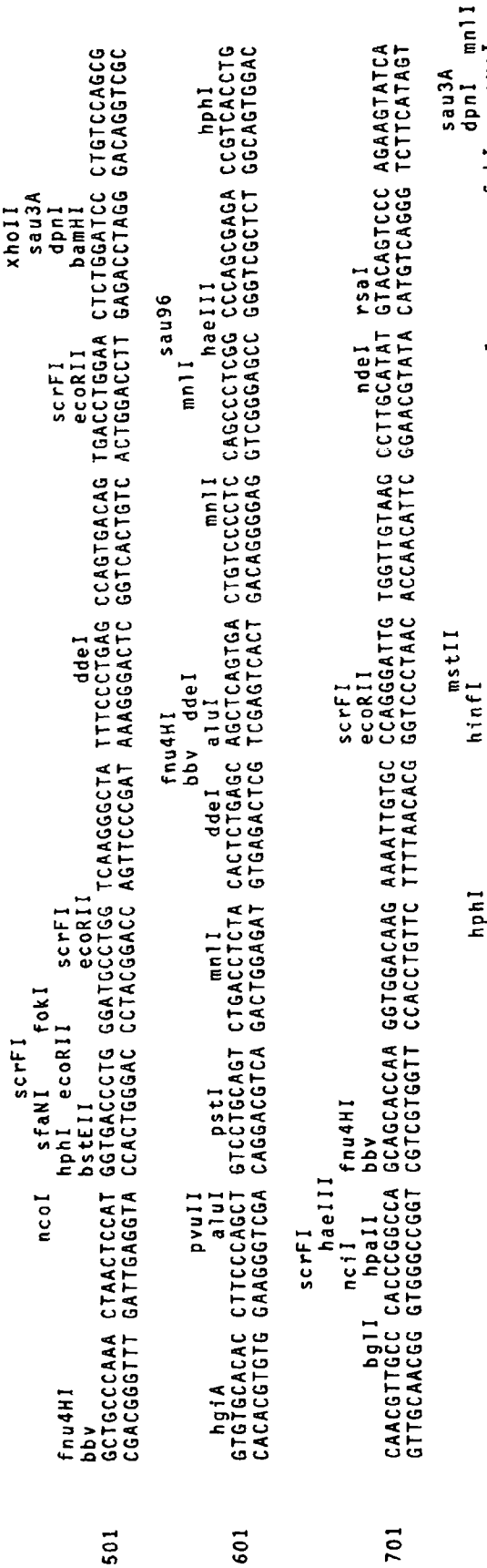

Fig. 4B.

```
                                                                                        fnu4HI
                    scrFI                                                               bbv
                    ecoRII                                                  hincII        aluI                  taqI
1001    ACTTCCCATC ATGCACCAGG ACTGGCTCAA TGGCAAGGAG TTCAAATGCA GGGTCAACAG TGCAGCTTTC CCTGCCCCCA TCGAGAAAAC CATCTCCAAA
        TGAAGGGTAG TACGTGGTCC TGACCGAGTT ACCGTTCCTC AAGTTTACGT CCCAGTTGTC ACGTCGAAAG GGACGGGGGT AGCTCTTTTG GTAGAGGTTT

                                                                                     haeIII
                                                                                   haeI
                                            rsaI           mnlI                    balI
1101    ACCAAAGGCA GACCGAAGGC TCCACAGGTG TACACCATTC CACCTCCCAA GGAGCAGATG GCCAAGGATA AAGTCAGTCT GACCTGCATG ATAACAGACT
        TGGTTTCCGT CTGGCTTCCG AGGTGTCCAC ATGTGGTAAG GTGGAGGGTT CCTCGTCTAC CGGTTCCTAT TTCAGTCAGA CTGGACGTAC TATTGTCTGA

                                                  fnu4HI
        mboII      mboII                          bbv                               ddeI
1201    TCTTCCCTGA AGACATTACT GTGGAGTGGC AGTGGAATGG GCAGCCAGCG GAGAACTACA AGAACACTCA GCCCATCATG AACACGAATG GCTCTTACTT
        AGAAGGGACT TCTGTAATGA CACCTCACCG TCACCTTACC CGTCGGTCGC CTCTTGATGT TCTTGTGAGT CGGGTAGTAC TTGTGCTTAC CGAGAATGAA

                                                                                                sau96
                                                                                              mnlI                    mboII
          accI         aluI           mboII           mnlI              hphI                    haeIII               ddeI
1301    CGTCTACAGC AAGCTCAATG TGCAGAAGAG CAACTGGGAG GCAGGAAATA CTTTCACCTG CTCTGTGTTA CATGAGGGCC TGCACAACCA CCATACTGAG
        GCAGATGTCG TTCGAGTTAC ACGTCTTCTC GTTGACCCTC CGTCCTTTAT GAAAGTGGAC GAGACACAAT GTACTCCCGG ACGTGTTGGT GGTATGACTC

                                 scrFI      sau3A                       sau96
            mnlI                 ecoRII     dpnI                  mnlI  avaII         hinfI          mnlI      mnlI
1401    AAGAGCCTCT CCCACTCTCC TGGTAAATGA TCCCAGTGTC CTTGGAGCCC TCTGGTCCTA CAGGACTCTG ACACCTACCT CCACCCCTCC CTGTATAAAT
        TTCTCGGAGA GGGTGAGAGG ACCATTTACT AGGGTCACAG GAACCTCGGG AGACCAGGAT GTCCTGAGAC TGTGGATGGA GGTGGGGAGG GACATATTTA

1501    AAAGCACCCA GCACTGCCTT GGGAAAAA
        TTTCGTGGGT CGTGACGGAA CCCTTTTT
```

Fig. 4C.

```
                                                                  -10                                 1
                             met asn phe gly leu ser leu ile tyr leu val leu val leu lys val val gln cys glu
GAGUCAGCACUGAACACGGACCCCUCACG AUG AAC UUC GGG CUC AGC UUG AUU UAC CUU GUC CUG GUU UUA AAA GUU GUC CAG UGU GAA

                 10                      20                             30
val met leu val glu ser gly gly val leu met glu pro gly gly ser leu lys leu ser cys ala ala ser gly phe thr phe ser arg
GUG AUG CUG GUG GAG UCU GGG GGA GUC UUA AUG GAG CCU GGA GGG UCC CUG AAA CUC UCC UGU GCA GCC UCU GGA UUC ACU UUC AGU AGA

                 40                      50                             60
tyr ala met ser trp val arg gln thr pro glu lys arg leu glu trp val ala thr ile ser ser gly gly ser ser his leu pro ser
UAU GCC AUG UCU UGG GUU CGC CAG ACU CCG GAG AAG AGG CUG GAG UGG GUC GCA ACC AUU AGU AGU GGU GGU AGU UCA CAC CUU CCA UCC

                 70                      80                             90
arg gln cys glu gly arg phe thr ile ser arg asp asn ala lys asn thr leu tyr leu gln met ser ser leu arg ser glu asp thr
AGA CAG UGU GAA GGG CGA UUC ACC AUC UCC AGA GAC AAU GCC AAG AAC ACC CUG UAC CUG CAA AUG AGC AGU CUG AGG UCU GAG GAC ACG

                100                     110                            120
ala met tyr tyr cys ala arg pro pro leu ile ser leu val ala asp tyr ala met asp tyr trp gly gln gly thr ser val thr val
GCC AUG UAU UAC UGU GCA AGA CCC CCU CUU AUU UCG UUA GUA GCG GAC UAU GCU AUG GAC UAC UGG GGU CAA GGA ACC UCA GUC ACC GUC

                130                     140                            150
ser ser ala lys thr thr pro pro ser val tyr pro leu ala pro gly ser ala ala gln thr asn ser met val thr leu gly cys leu
UCC UCA GCC AAA ACG ACA CCC CCA UCU GUC UAU CCA CUG GCC CCU GGA UCU GCU GCC CAA ACU AAC UCC AUG GUG ACC CUG GGA UGC CUG

                160                     170                            180
val lys gly tyr phe pro glu pro val thr val thr trp asn ser gly ser leu ser ser gly val his thr phe pro ala val leu gln
GUC AAG GGC UAU UUC CCU GAG CCA GUG ACA GUG ACC UGG AAC UCU GGA UCC CUG UCC AGC GGU GUG CAC ACC UUC CCA GCU GUC CUG CAG

                190                     200                            210
ser asp leu tyr thr leu ser ser ser val thr val pro ser ser pro arg pro ser glu thr val thr cys asn val ala his pro ala
UCU GAC CUC UAC ACU CUG AGC AGC UCA GUG ACU GUC CCC UCC AGC CCU CGG CCC AGC GAG ACC GUC ACC UGC AAC GUU GCC CAC CCG GCC

                220                     230                            240
ser ser thr lys val asp lys lys ile val pro arg asp cys gly cys lys pro cys ile cys thr val pro glu val ser ser val phe
AGC AGC ACC AAG GUG GAC AAG AAA AUU GUG CCC AGG GAU UGU GGU UGU AAG CCU UGC AUA UGU ACA GUC CCA GAA GUA UCA UCU GUC UUC
```

Fig. 5A.

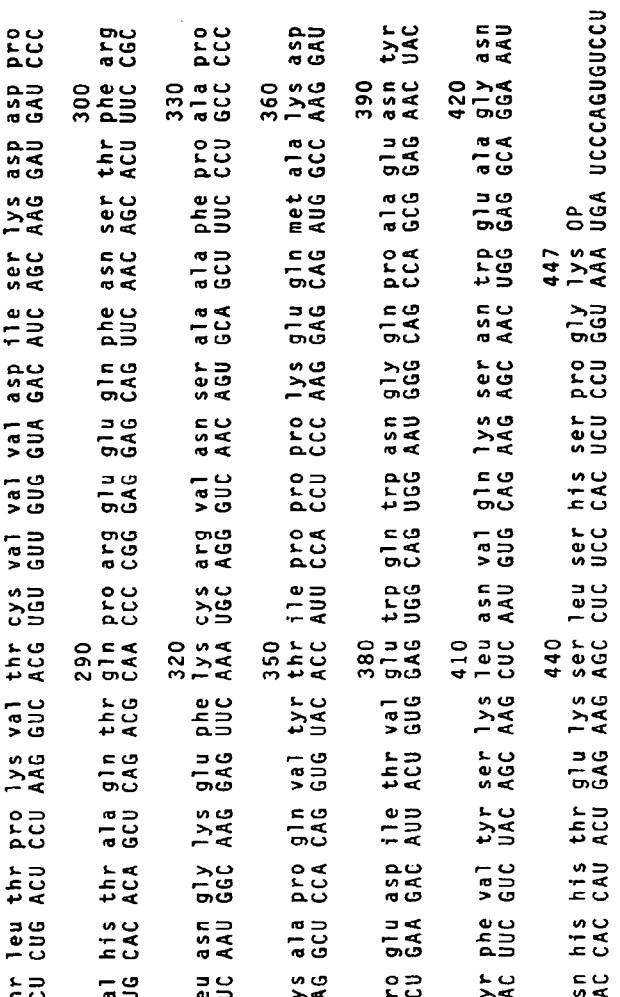

Fig. 5B.

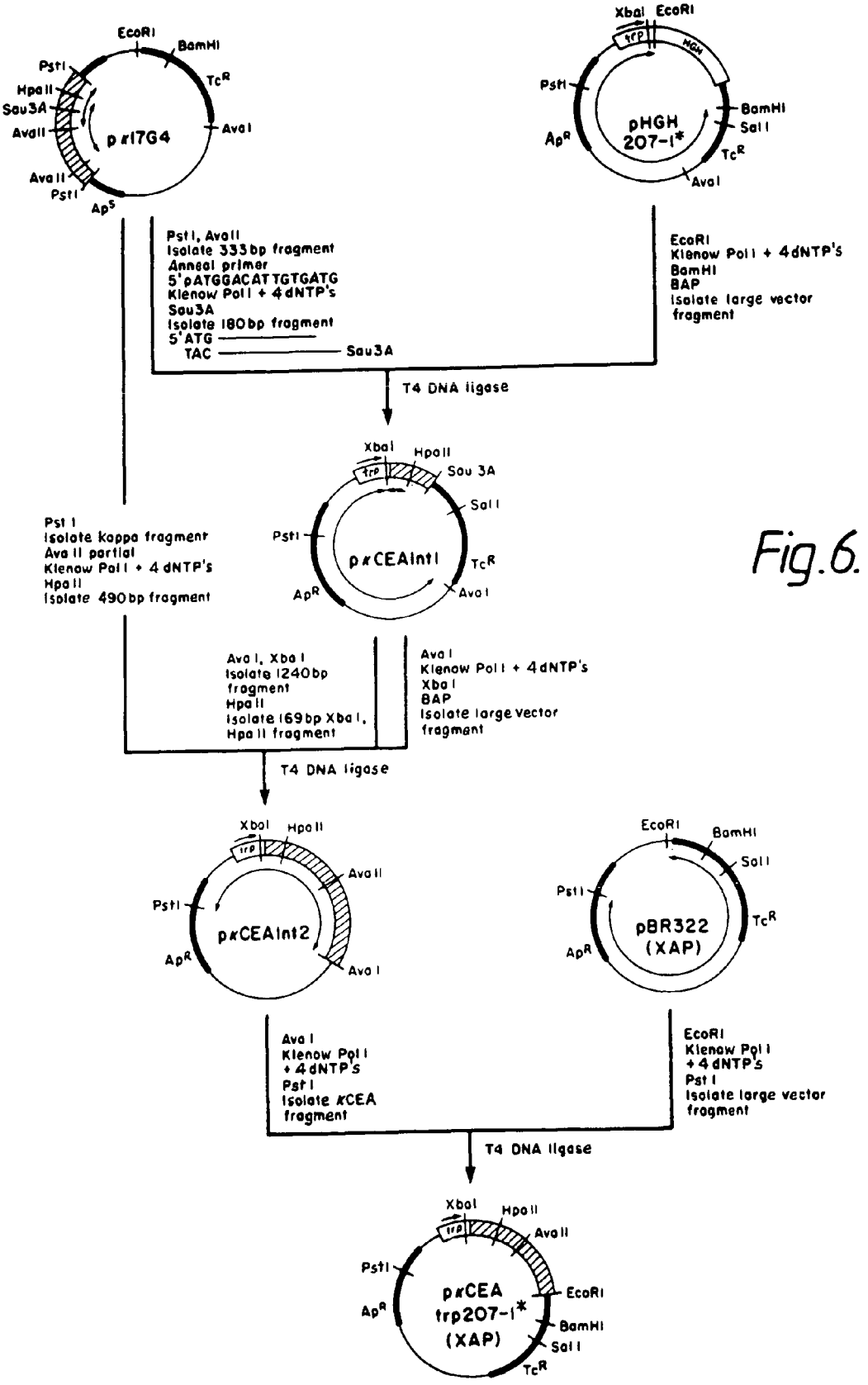
EcoRI
BamHI
PstI
HpaII
Sau3A
AvaII
TcR
AvaI
pκ17G4
ApS
XbaI
EcoRI
trp
HGH
PstI
pHGH
207-1*
BamHI
SalI
ApR
TcR
AvaI
PstI, AvaII
Isolate 333bp fragment
Anneal primer
5'pATGGACATTGTGATG
Klenow PolI + 4dNTP's
Sau3A
Isolate 180bp fragment
5'ATG
TAC
Sau3A
EcoRI
Klenow PolI + 4dNTP's
BamHI
BAP
Isolate large vector fragment
T4 DNA ligase
XbaI
HpaII
trp
Sau 3A
SalI
PstI
pκCEAint1
TcR
ApR
AvaI
Pst I
Isolate kappa fragment
Ava II partial
Klenow PolI + 4 dNTP's
Hpa II
Isolate 490bp fragment
AvaI, XbaI
Isolate 1240bp fragment
HpaII
Isolate 169bp XbaI, HpaII fragment
AvaI
Klenow PolI + 4dNTP's
XbaI
BAP
Isolate large vector fragment
T4 DNA ligase
XbaI
HpaII
trp
AvaII
PstI
pκCEAint2
ApR
AvaI
EcoRI
BamHI
SalI
PstI
pBR322
(XAP)
TcR
ApR
AvaI
Klenow PolI
+4dNTP's
PstI
Isolate κCEA fragment
EcoRI
Klenow PolI
+4dNTP's
PstI
Isolate large vector fragment
T4 DNA ligase
XbaI
HpaII
trp
AvaII
PstI
pκCEA
trp207-1*
(XAP)
EcoRI
BamHI
SalI
ApR
TcR

Fig. 6.

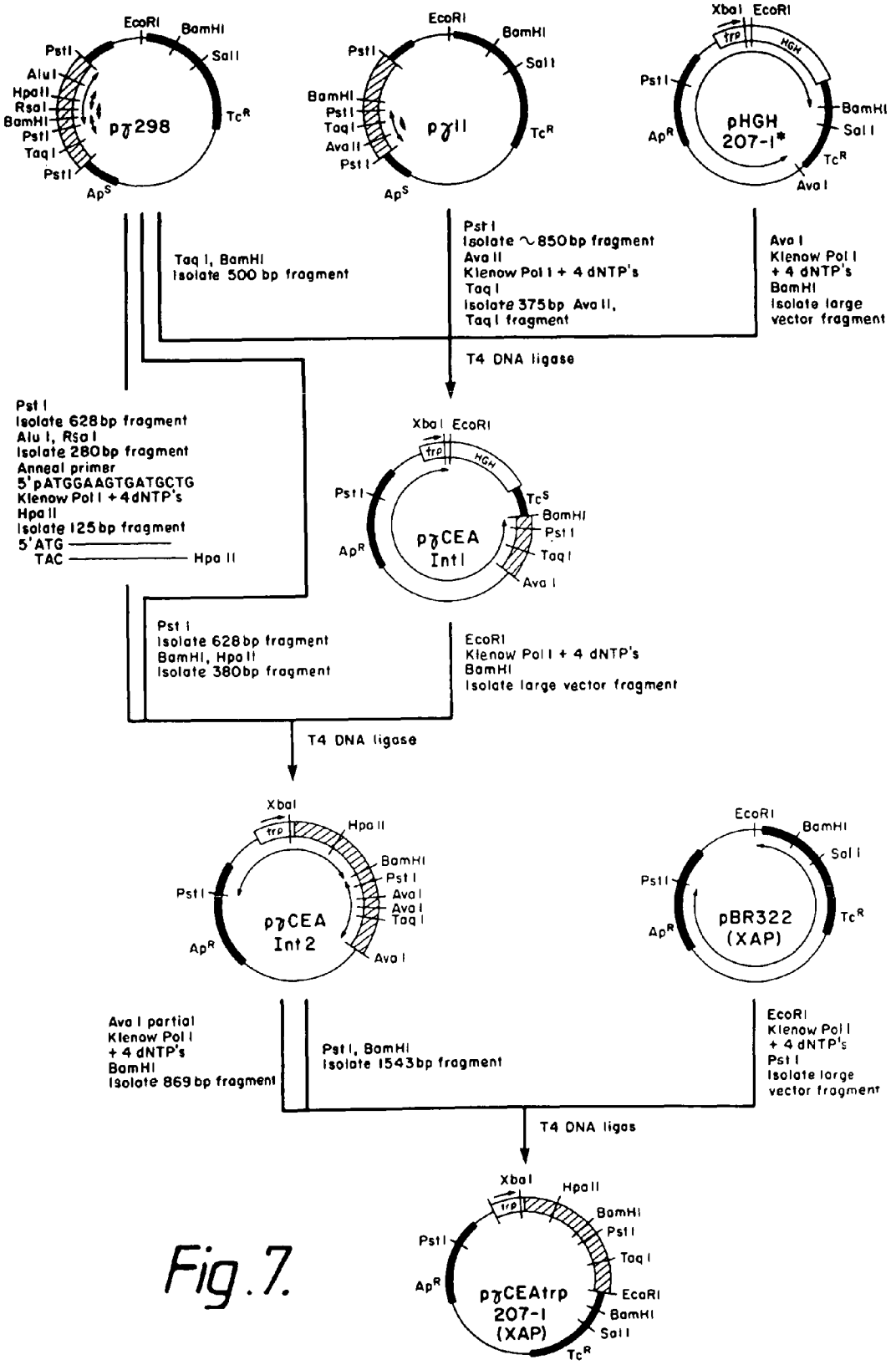
pγ298
pγII
pHGH 207-1*
pγCEA Int1
pγCEA Int2
pBR322 (XAP)
pγCEAtrp 207-1 (XAP)
Taq I, BamHI Isolate 500 bp fragment
Pst I Isolate ~850bp fragment Ava II Klenow Pol I + 4 dNTP's Taq I Isolate 375bp Ava II, Taq I fragment
Ava I Klenow Pol I + 4 dNTP's BamHI Isolate large vector fragment
T4 DNA ligase
Pst I Isolate 628bp fragment Alu I, Rsa I Isolate 280bp fragment Anneal primer 5'pATGGAAGTGATGCTG Klenow Pol I + 4dNTP's Hpa II Isolate 125bp fragment 5'ATG TAC Hpa II
Pst I Isolate 628bp fragment BamHI, Hpa II Isolate 380bp fragment
EcoRI Klenow Pol I + 4 dNTP's BamHI Isolate large vector fragment
T4 DNA ligase
Ava I partial Klenow Pol I + 4 dNTP's BamHI Isolate 869 bp fragment
Pst I, BamHI Isolate 1543bp fragment
EcoRI Klenow Pol I + 4 dNTP's Pst I Isolate large vector fragment
T4 DNA ligas

Fig. 7.

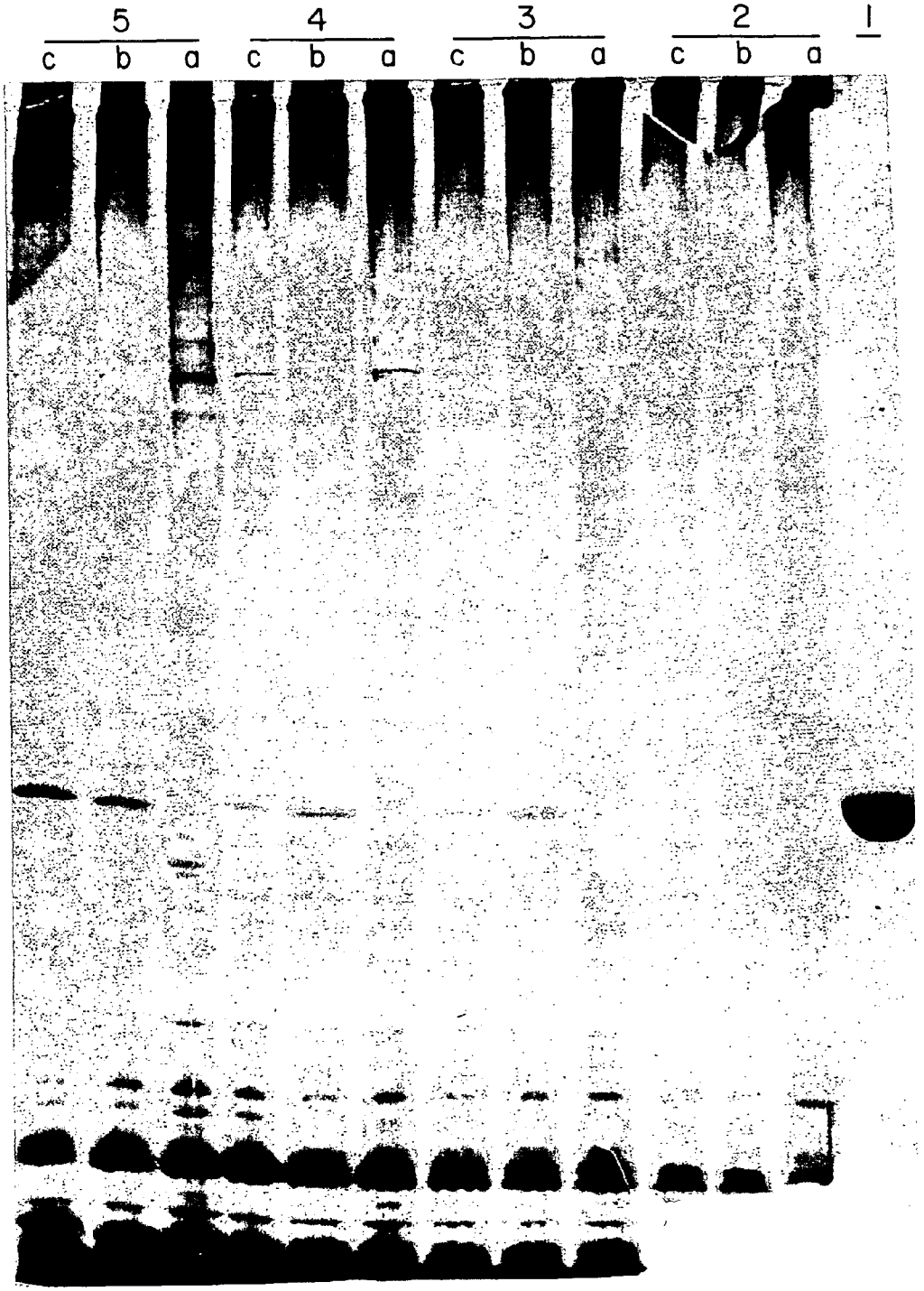

Fig. 8A.

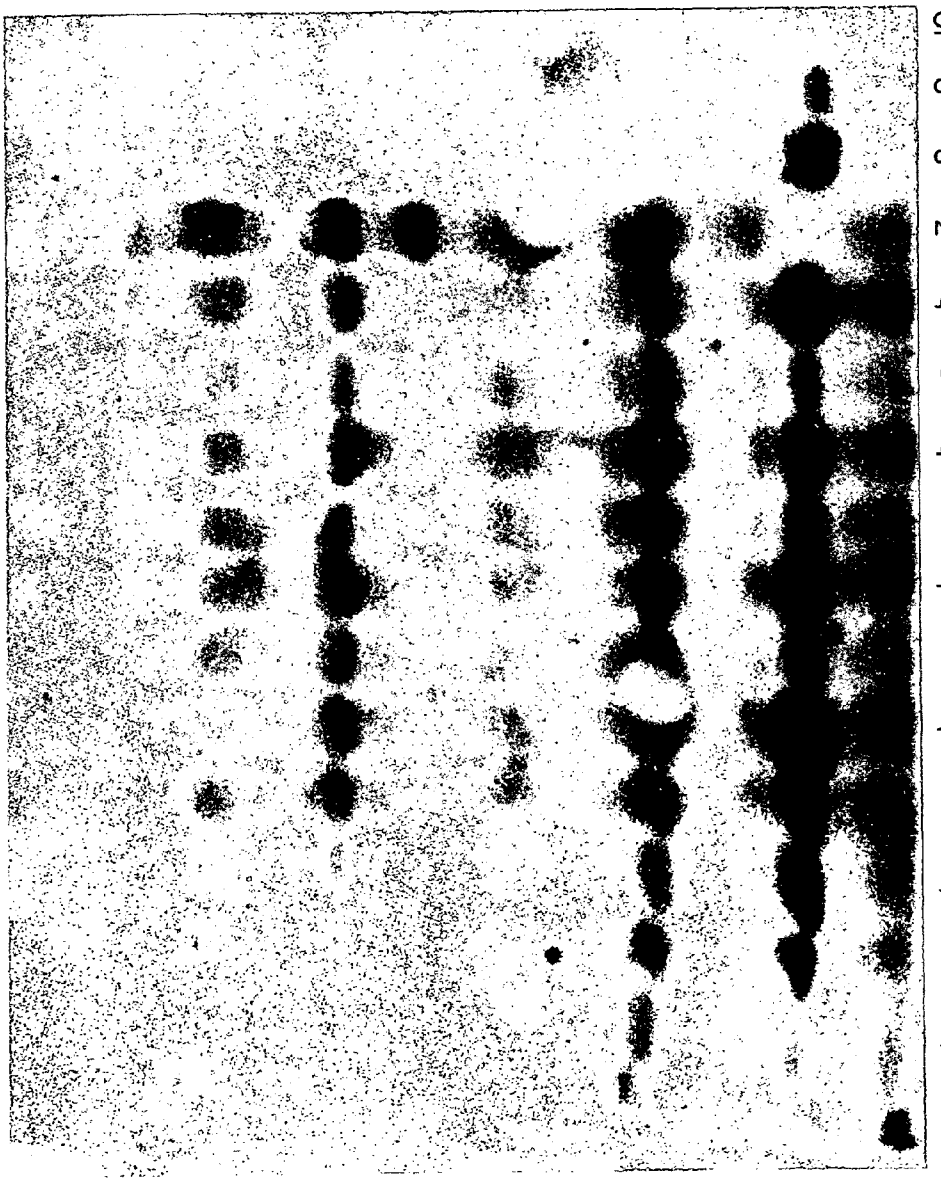

Fig.8B.

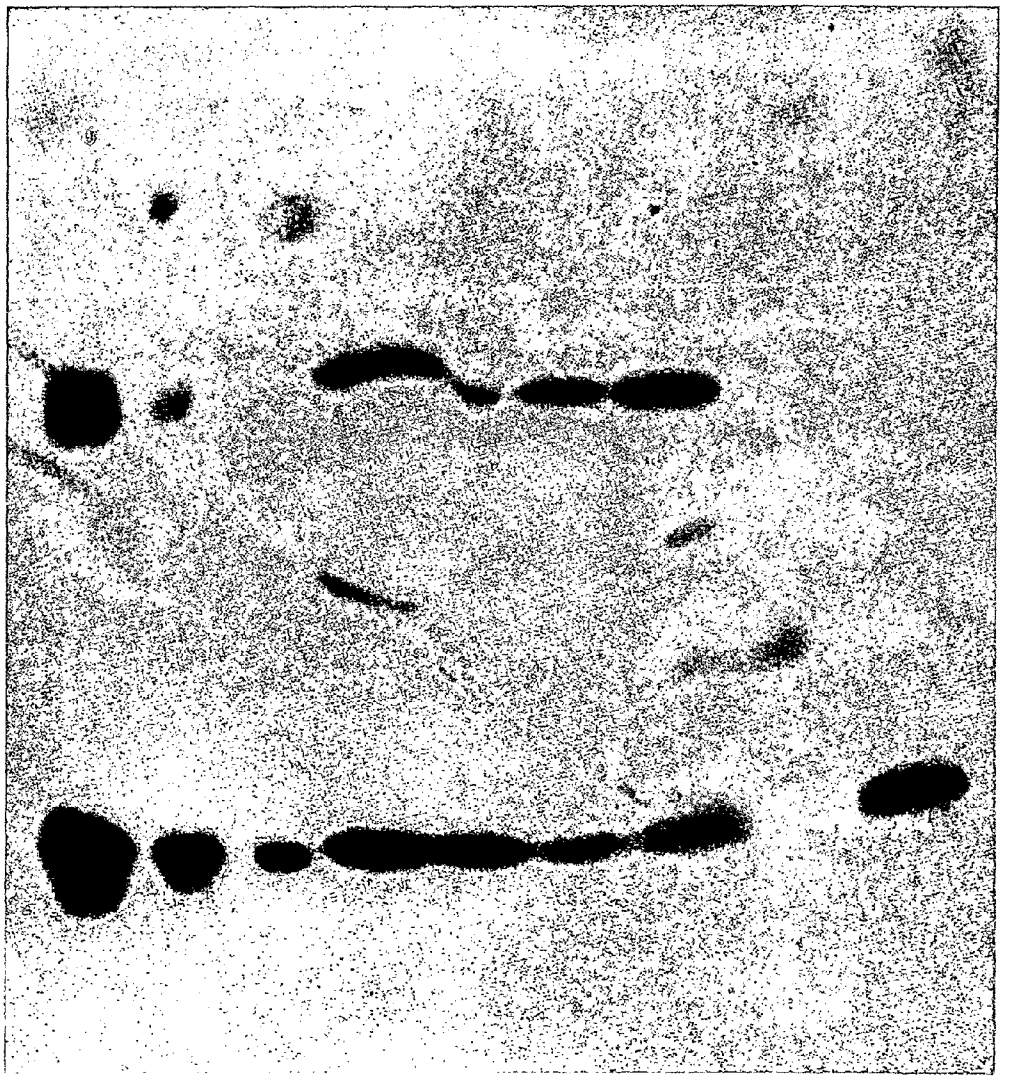

Fig. 8C.

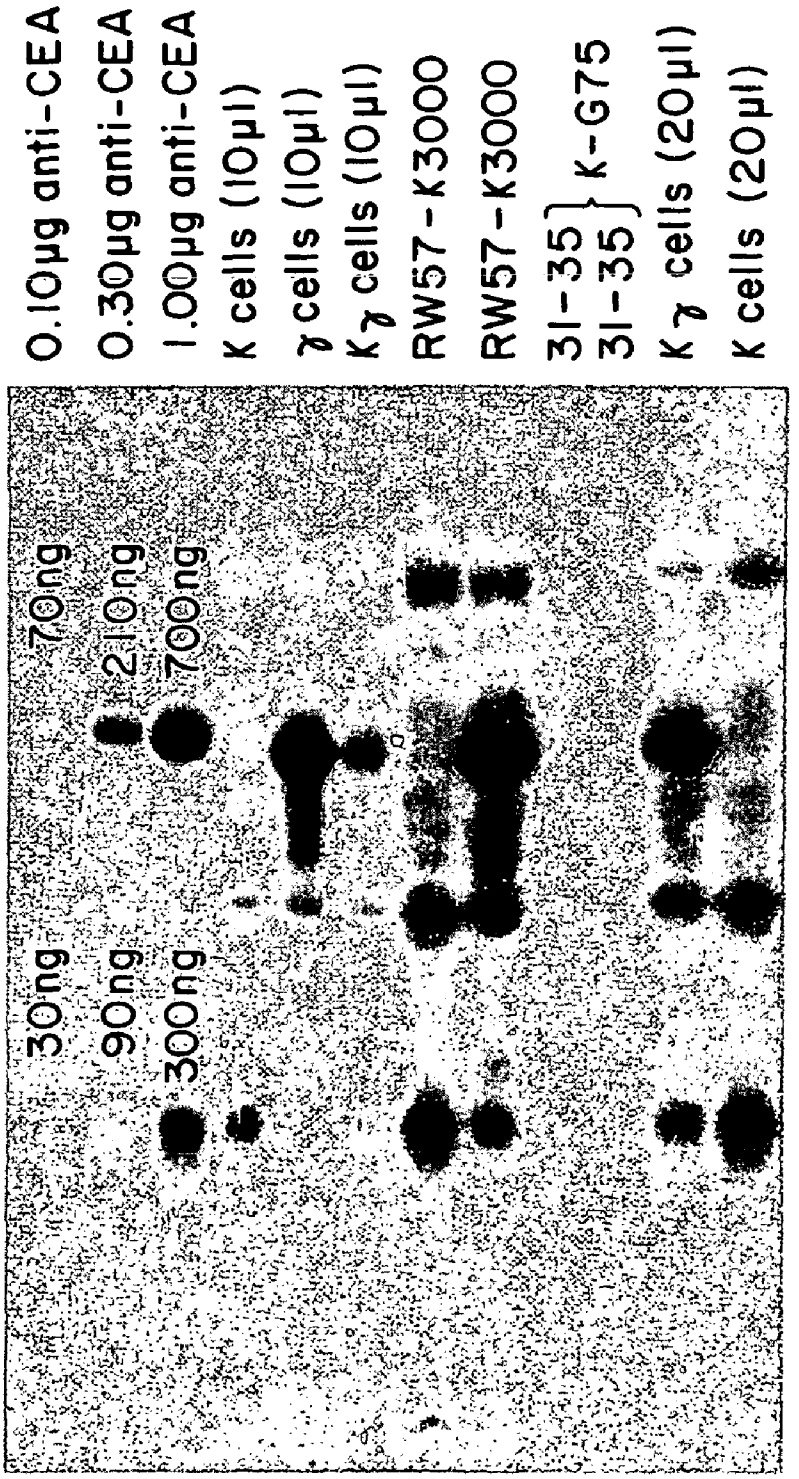
0.10µg anti-CEA
0.30µg anti-CEA
1.00µg anti-CEA
K cells (10µl)
γ cells (10µl)
Kγ cells (10µl)
RW57-K3000
RW57-K3000
31-35 } K-G75
31-35 }
Kγ cells (20µl)
K cells (20µl)
70ng
210ng
700ng
30ng
90ng
300ng

Fig. 9.

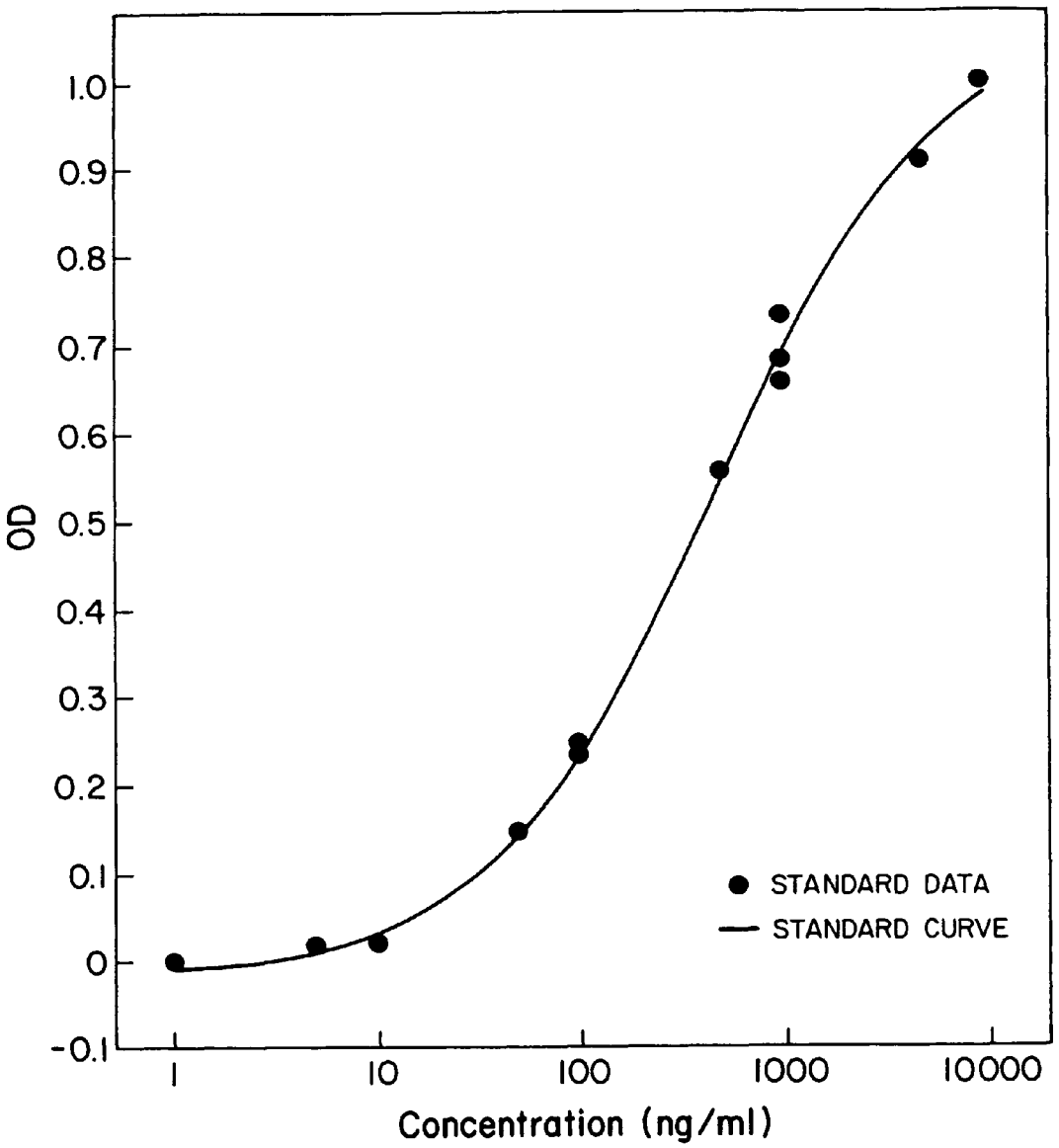
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
OD
1
10
100
1000
10000
Concentration (ng/ml)
STANDARD DATA
STANDARD CURVE

Fig. 10.

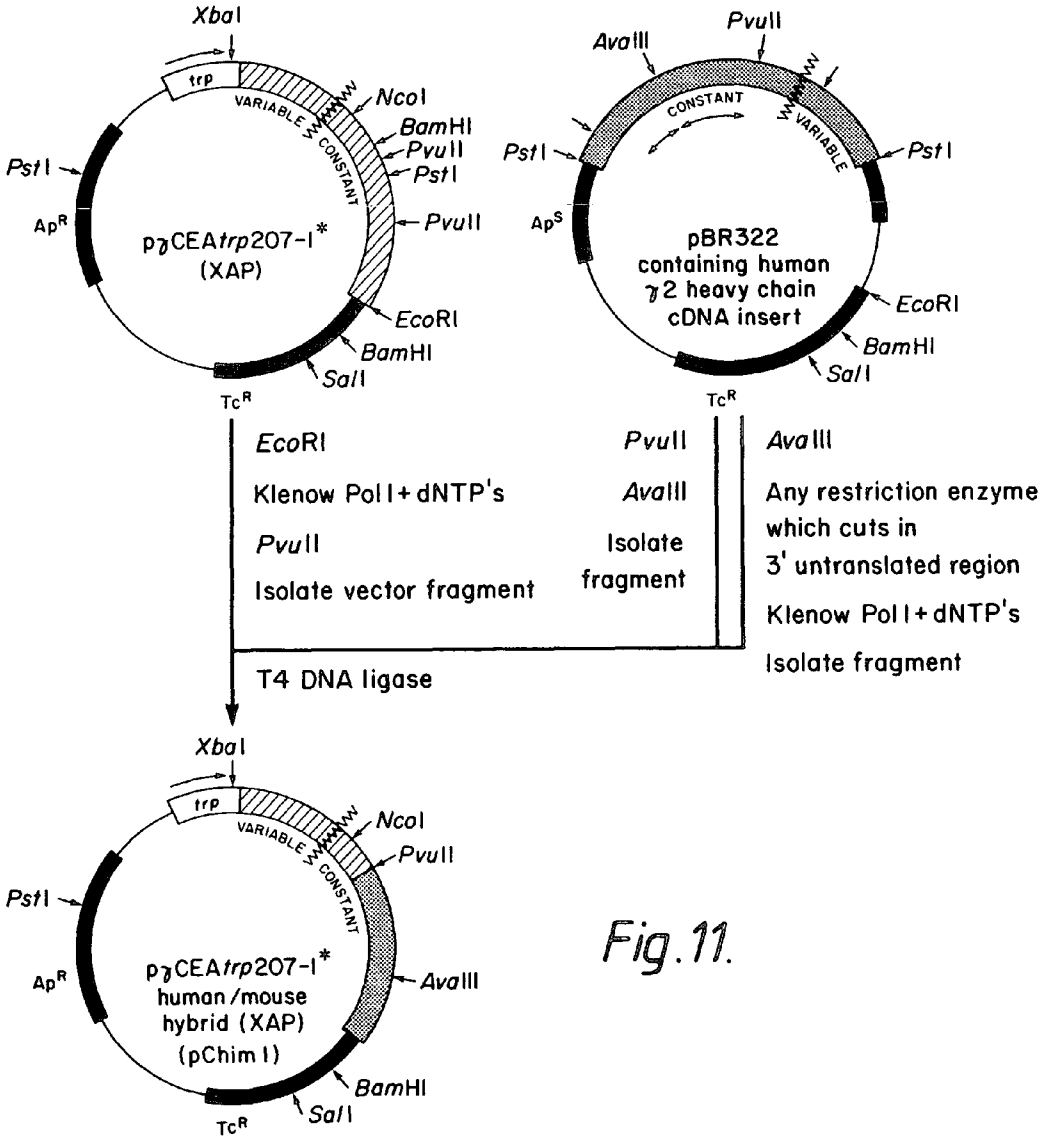
XbaI
trp
VARIABLE
CONSTANT
NcoI
BamHI
PvuII
PstI
PvuII
EcoRI
BamHI
SalI
TcR
PstI
ApR
pγCEAtrp207-1*
(XAP)
AvaIII
PvuII
CONSTANT
VARIABLE
PstI
PstI
ApS
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
TcR
EcoRI
Klenow PolI+dNTP's
PvuII
Isolate vector fragment
PvuII
AvaIII
Isolate
fragment
AvaIII
Any restriction enzyme
which cuts in
3' untranslated region
Klenow PolI+dNTP's
Isolate fragment
T4 DNA ligase
XbaI
trp
VARIABLE
CONSTANT
NcoI
PvuII
PstI
ApR
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim1)
AvaIII
BamHI
SalI
TcR

Fig. 11.

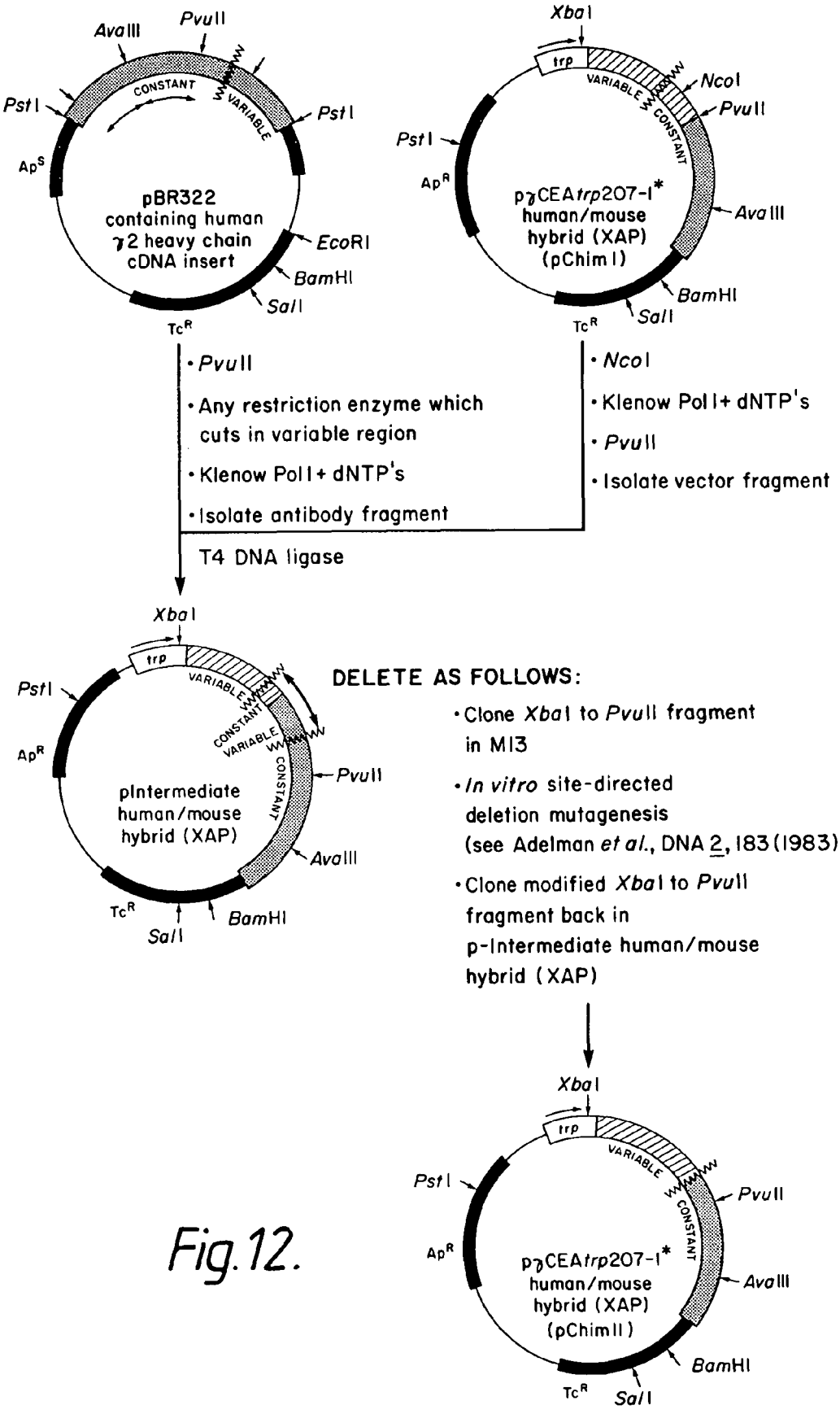
AvaIII
PvuII
PstI
CONSTANT
VARIABLE
PstI
Ap^S
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
Tc^R
XbaI
trp
VARIABLE
NcoI
PvuII
PstI
Ap^R
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim1)
AvaIII
BamHI
SalI
Tc^R
• PvuII
• Any restriction enzyme which cuts in variable region
• Klenow Pol I + dNTP's
• Isolate antibody fragment
• NcoI
• Klenow Pol I + dNTP's
• PvuII
• Isolate vector fragment
T4 DNA ligase
XbaI
trp
VARIABLE
CONSTANT
VARIABLE
CONSTANT
PstI
Ap^R
pIntermediate
human/mouse
hybrid (XAP)
PvuII
AvaIII
Tc^R
SalI
BamHI
DELETE AS FOLLOWS:
• Clone XbaI to PvuII fragment in M13
• In vitro site-directed deletion mutagenesis (see Adelman et al., DNA 2, 183 (1983))
• Clone modified XbaI to PvuII fragment back in p-Intermediate human/mouse hybrid (XAP)
XbaI
trp
VARIABLE
CONSTANT
PstI
PvuII
Ap^R
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim11)
AvaIII
BamHI
Tc^R
SalI

Fig. 12.

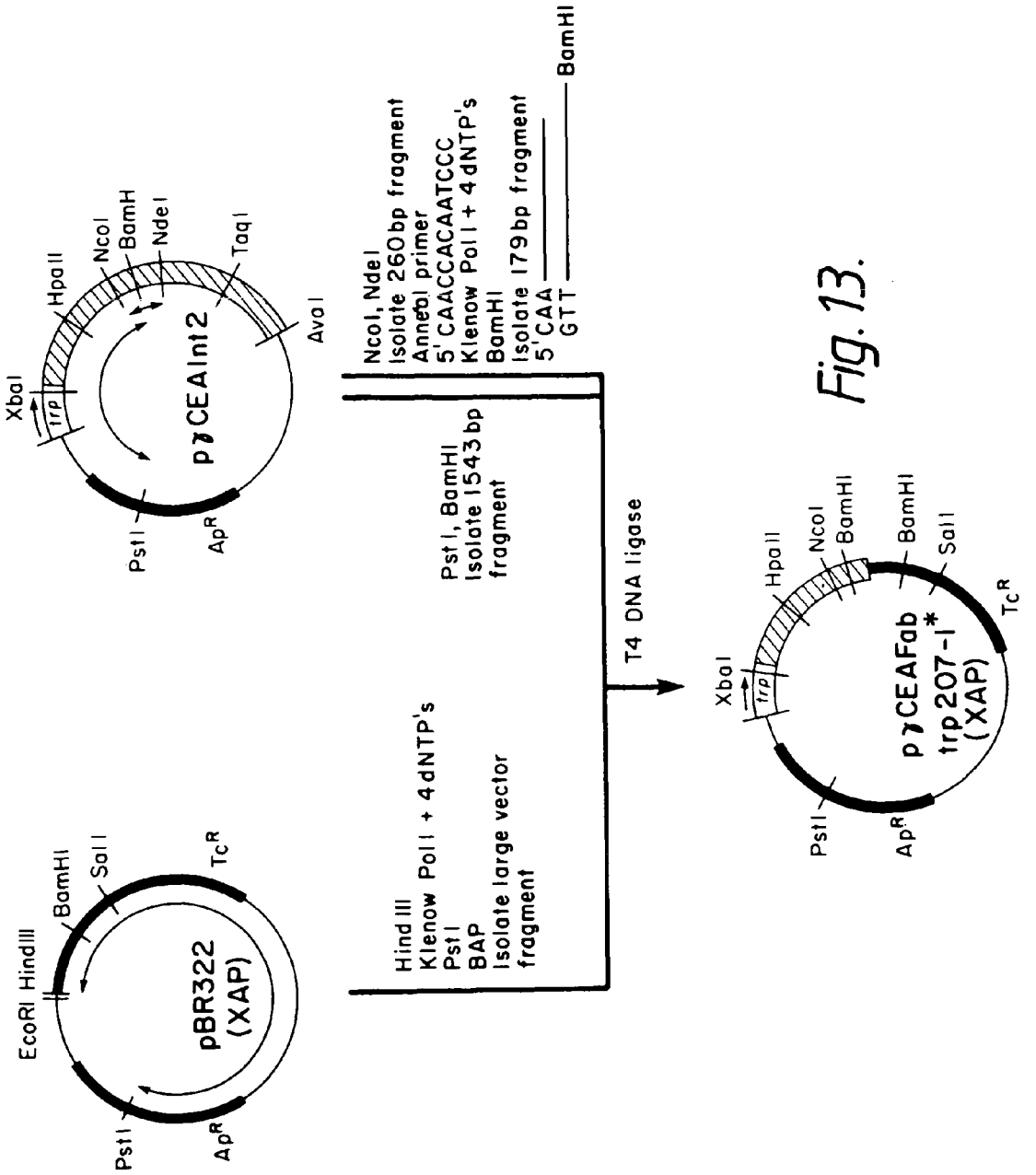
pBR322 (XAP)
EcoRI HindIII
BamHI
SalI
TcR
PstI
ApR
Hind III
Klenow Pol I + 4 dNTP's
PstI
BAP
Isolate large vector fragment
pγCEAIn12
XbaI
trp
HpaII
NcoI
BamH
NdeI
TaqI
AvaI
PstI
ApR
PstI, BamHI
Isolate 1543 bp
fragment
NcoI, NdeI
Isolate 260bp fragment
Anneal primer
5' CAACCACAATCCC
Klenow Pol I + 4 dNTP's
BamHI
Isolate 179bp fragment
5' CAA
GTT
BamHI
T4 DNA ligase
pγCEAFab trp207-1* (XAP)
XbaI
trp
HpaII
NcoI
BamHI
BamHI
SalI
TcR
PstI
ApR

Fig. 13.

METHODS OF MAKING ANTIBODY HEAVY AND LIGHT CHAINS HAVING SPECIFICITY FOR A DESIRED ANTIGEN

This is a continuation of application(s) Ser. No. 07/205,419 filed on 10 Jun. 1988, now U.S. Pat. No. 6,331,415, issued on 18 Dec. 2001, which is a continuation of Ser. No. 06/483,457 filed on 8 Apr. 1983, now U.S. Pat. No. 4,816,567, issued on 28 Mar. 1989, which applications are incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.

BACKGROUND OF THE INVENTION

This invention relates to the field of immunoglobulin production and to modification of naturally occurring immunoglobulin amino acid sequences. Specifically, the invention relates to using recombinant techniques to produce both immunoglobulins which are analogous to those normally found in vertebrate systems and to take advantage of these gene modification techniques to construct chimeric or other modified forms.

A. Immunoglobulins and Antibodies

Antibodies are specific immunoglobulin polypeptides produced by the vertebrate immune system in response to challenge by foreign proteins, glycoproteins, cells, or other antigenic foreign substances. The sequence of events which permits the organism to overcome invasion by foreign cells or to rid the system of foreign substances is at least partially understood. An important part of this process is the manufacture of antibodies which bind specifically to a particular foreign substance. The binding specificity of such polypeptides to a particular antigen is highly refined, and the multitude of specificities capable of being generated by the individual vertebrate is remarkable in its complexity and variability. Thousands of antigens are capable of eliciting responses, each almost exclusively directed to the particular antigen which elicited it.

Immunoglobulins include both antibodies, as above described, and analogous protein substances which lack antigen specificity. The latter are produced at low levels by the lymph system and in increased levels by myelomas.

A.1 Source and Utility

Two major sources of vertebrate antibodies are presently utilized—generation in situ by the mammalian B lymphocytes and in cell culture by B-cell hybrids. Antibodies are made in situ as a result of the differentiation of immature B lymphocytes into plasma cells, which occurs in response to stimulation by specific antigens. In the undifferentiated B cell, the portions of DNA coding for the various regions on the immunoglobulin chains are separated in the genomic DNA. The sequences are reassembled sequentially prior to transcription. A review of this process has been given by Gough, *Trends in Biochem Sci,* 6: 203 (1981). The resulting rearranged genome is capable of expression in the mature B lymphocyte to produce the desired antibody. Even when only a single antigen is introduced into the sphere of the immune system for a particular mammal, however, a uniform population of antibodies does not result. The in situ immune response to any particular antigen is defined by the mosaic of responses to the various determinants which are present on the antigen. Each subset of homologous antibody is contributed by a single population of B cells—hence in situ generation of antibodies is "polyclonal".

This limited but inherent heterogeneity has been overcome in numerous particular cases by use of hybridoma technology to create "monoclonal" antibodies (Kohler, et al., *Eur. J. Immunol.,* 6: 511 (1976)). In this process, splenocytes or lymphocytes from a mammal which has been injected with antigen are fused with a tumor cell line, thus producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell. The hybrids thus formed are segregated into single genetic strains by selection, dilution, and regrowth, and each strain thus represents a single genetic line. They therefore produce immunoreactive antibodies against a desired antigen which are assured to be homogenous, and which antibodies, referencing their pure genetic parentage, are called "monoclonal". Hybridoma technology has to this time been focused largely on the fusion of murine lines, but human-human hybridomas (Olsson, L. et al., *Proc. Natl. Acad. Sci. (USA),* 77: 5429 (1980)); human-murine hybridomas (Schlom, J., et al. (ibid) 77: 6841 (1980)) and several other xenogenic hybrid combinations have been prepared as well. Alternatively, primary, antibody producing, B cells have been immortalized in vitro by transformation with viral DNA.

Polyclonal, or, much more preferably, monoclonal, antibodies have a variety of useful properties similar to those of the present invention. For example, they can be used as specific immunoprecipitating reagents to detect the presence of the antigen which elicited the initial processing of the B cell genome by coupling this antigen-antibody reaction with suitable detection techniques such as labeling with radioisotopes or with enzymes capable of assay (RIA, EMIT, and ELISA). Antibodies are thus the foundation of immuno diagnostic tests for many antigenic substances. In another important use, antibodies can be directly injected into subjects suffering from an attack by a substance or organism containing the antigen in question to combat this attack. This process is currently in its experimental stages, but its potential is clearly seen. Third, whole body diagnosis and treatment is made possible because injected antibodies are directed to specific target disease tissues, and thus can be used either to determine the presence of the disease by carrying with them a suitable label, or to attack the diseased tissue by carrying a suitable drug.

Monoclonal antibodies produced by hybridomas, while theoretically effective as suggested above and clearly preferable to polyclonal antibodies because of their specificity, suffer from certain disadvantages. First, they tend to be contaminated with other proteins and cellular materials of hybridoma, (and, therefore, mammalian) origin. These cells contain additional materials, notably nucleic acid fragments, but protein fragments as well, which are capable of enhancing, causing, or mediating carcinogic responses. Second, hybridoma lines producing monoclonal antibodies tend to be unstable and may alter the structure of antibody produced or stop producing antibody altogether (Kohler, G., et al., *Proc. Natl. Acad. Sci (USA)* 77: 2197 (1980); Morrison, S. L., *J. Immunol.* 123: 793 (1979)). The cell line genome appears to alter itself in response to stimuli whose nature is not currently known, and this alteration may result in production of incorrect sequences. Third, both hybridoma and B cells inevitably produce certain antibodies in glycosylated form (Melchers, F., *Biochemistry,* 10: 653 (1971)) which, under some circumstances, may be undesirable. Fourth, production of both monoclonal and polyclonal antibodies is relatively expensive. Fifth, and perhaps most important, production by current techniques (either by hybridoma or by B cell response) does not permit manipulation of the genome so as to produce antibodies with more effective design components than those normally elicited in response to antigens from the mature B cell in situ. The antibodies of the present invention do not

suffer from the foregoing drawbacks, and, furthermore, offer the opportunity to provide molecules of superior design.

Even those immunoglobulins which lack the specificity of antibodies are useful, although over a smaller spectrum of potential uses than the antibodies themselves. In presently understood applications, such immunoglobulins are helpful in protein replacement therapy for globulin related anemia. In this context, an inability to bind to antigen is in fact helpful, as the therapeutic value of these proteins would be impaired by such functionality. At present, such non-specific antibodies are derivable in quantity only from myeloma cell cultures suitably induced. The present invention offers an alternative, more economical source. It also offers the opportunity of cancelling out specificity by manipulating the four chains of the tetramer separately.

A.2 General Structure Characteristics

The basic immunoglobin structural unit in vertebrate systems is now well understood (Edelman, G. M., *Ann. N.Y. Acad. Sci.,* 190: 5 (1971)). The units are composed of two identical light polypeptide chains of molecular weight approximately 23,000 daltons, and two identical heavy chains of molecular weight 53,000-70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the Y and continuing through the divergent region as shown in FIG. **1**. The "branch" portion, as there indicated, is designated the Fab region. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, with some subclasses among them, and the nature of this chain, as it has a long constant region, determines the "class" of the antibody as IgG, IgM, IgA, IgD, or IgE. Light chains are classified as either kappa or lambda. Each heavy chain class can be prepared with either kappa or lambda light chain. The light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells. However, if non-covalent association of the chains can be effected in the correct geometry, the aggregate will still be capable of reaction with antigen, or of utility as a protein supplement as a non-specific immunoglobulin.

The amino acid sequence runs from the N-terminal end at the top of the Y to the C-terminal end at the bottom of each chain. At the N-terminal end is a variable region which is specific for the antigen which elicited it, and is approximately 100 amino acids in length, there being slight variations between light and heavy chain and from antibody to antibody. The variable region is linked in each chain to a constant region which extends the remaining length of the chain. Linkage is seen, at the genomic level, as occurring through a linking sequence known currently as the "J" region in the light chain gene, which encodes about 12 amino acids, and as a combination of "D" region and "J" region in the heavy chain gene, which together encode approximately 25 amino acids.

The remaining portions of the chain are referred to as constant regions and within a particular class do not to vary with the specificity of the antibody (i.e., the antigen eliciting it).

As stated above, there are five known Major classes of constant regions which determine the class of the immunoglobulin molecule (IgG, IgM, IgA, IgD, and IgE corresponding to γ, μ, α, δ, and ϵ heavy chain constant regions). The constant region or class determines subsequent effector function of the antibody, including activation of complement (Kabat, E. A., *Structural Concepts in Immunology and Immunochemistry,* 2nd Ed., p. 413-436, Holt, Rinehart, Winston (1976)), and other cellular responses (Andrews, D. W., et al., *Clinical Immunobiology* pp 1-18, W. B. Sanders (1980); Kohl, S., et al., *Immunology,* 48: 187 (1983)); while the variable region determines the antigen with which it will react.

B. Recombinant DNA Technology

Recombinant DNA technology has reached sufficient sophistication that it includes a repertoire of techniques for cloning and expression of gene sequences. Various DNA sequences can be recombined with some facility, creating new DNA entities capable of producing heterologous protein product in transformed microbes and cell cultures. The general means and methods for the in vitro ligation of various blunt ended or "sticky" ended fragments of DNA, for producing expression vectors, and for transforming organisms are now in hand.

DNA recombination of the essential elements (i.e., an origin of replication, one or more phenotypic selection characteristics, expression control sequence, heterologous gene insert and remainder vector) generally is performed outside the host cell. The resulting recombinant replicable expression vector, or plasmid, is introduced into cells by transformation and large quantities of the recombinant vehicle is obtained by growing the transformant. Where the gene is properly inserted with reference to portions which govern the transcription and translation of the encoded DNA message, the resulting expression vector is useful to produce the polypeptide sequence for which the inserted gene codes, a process referred to as "expression." The resulting product may be obtained by lysis, if necessary, of the host cell and recovery of the product by appropriate purifications from other proteins.

In practice, the use of recombinant DNA technology can express entirely heterologous polypeptides—so-called direct expression—or alternatively may express a heterologous polypeptide fused to a portion of the amino acid sequence of a homologous polypeptide. In the latter cases, the intended bioactive product is sometimes rendered bioinactive within the fused, homologous/heterologous polypeptide until it is cleaved in an extracellular environment.

The art of maintaining cell or tissue cultures as well as microbial systems for studying genetics and cell physiology is well established. Means and methods are available for maintaining permanent cell lines, prepared by successive serial transfers from isolated cells. For use in research, such cell lines are maintained on a solid support in liquid medium, or by growth in suspension containing support nutriments. Scale-up for large preparations seems to pose only mechanical problems.

SUMMARY OF THE INVENTION

The invention relates to antibodies and to non-specific immunoglobulins (NSIs) formed by recombinant techniques using suitable host cell cultures. These antibodies and NSIs can be readily prepared in pure "monoclonal" form. They can be manipulated at the genomic level to produce chimeras of variants which draw their homology from species which differ from each other. They can also be manipulated at the protein level, since all four chains do not need to be produced by the same cell. Thus, there are a number of "types" of immunoglobulins encompassed by the invention.

First, immunoglobulins, particularly antibodies, are produced using recombinant techniques which mimic the amino acid sequence of naturally occurring antibodies produced by either mammalian B cells in situ, or by B cells fused with suitable immortalizing tumor lines, i.e., hybridomas. Second, the methods of this invention produce, and the invention is

directed to, immunoglobulins which comprise polypeptides not hitherto found associated with each other in nature. Such reassembly is particularly useful in producing "hybrid" antibodies capable of binding more than one antigen; and in producing "composite" immunoglobuins wherein heavy and light chains of different origins essentially damp out specificity. Third, by genetic manipulation, "chimeric" antibodies can be formed wherein, for example, the variable regions correspond to the amino acid sequence from one mammalian model system, whereas the constant region mimics the amino acid sequence of another. Again, the derivation of these two mimicked sequences may be from different species. Fourth, also by genetic manipulation, "altered" antibodies with improved specificity and other characteristics can be formed.

Two other types of immunoglobulin-like moieties may be produced: "univalent" antibodies, which are useful as homing carriers to target tissues, and "Fab proteins" which include only the "Fab" region of an immunoglobulin molecule i.e, the branches of the "Y". These univalent antibodies and Fab fragments may also be "mammalian" i.e., mimic mammalian amino acid sequences; novel assemblies of mammalian chains, or chimeric, where for example, the constant and variable sequence patterns may be of different origin. Finally, either the light chain or heavy chain alone, or portions thereof, produced by recombinant techniques are included in the invention and may be mammalian or chimeric.

In other aspects, the invention is directed to DNA which encodes the aforementioned NSIs, antibodies, and portions thereof, as well as expression vectors or plasmids capable of effecting the production of such immunoglobulins in suitable host cells. It includes the host cells and cell cultures which result from transformation with these vectors. Finally, the invention is directed to methods of producing these NSIs and antibodies, and the DNA sequences, plasmids, and transformed cells intermediate to them.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a representation of the general structure of immunoglobulins.

FIGS. **2**A-B show the detailed sequence of the cDNA insert of pK17G4 which encodes kappa anti CEA chain.

FIG. **3** shows the coding sequence of the fragment shown in FIG. **2**, along with the corresponding amino acid sequence.

FIGS. **4**A-C show the combined detailed sequence of the cDNA inserts of pγ298 and pγ11 which encode gamma anti CEA chain.

FIGS. **5**A-B show the corresponding amino acid sequence encoded by the fragment in FIG. **4**.

FIGS. **6** and **7** outline the construction of expression vectors for kappa and gamma anti-CEA chains respectively.

FIGS. **8**A, **8**B, and **8**C show the results of sizing gels run on extracts of *E. coli* expressing the genes for gamma chain, kappa chain, and both kappa and gamma chains respectively.

FIG. **9** shows the results of western blots of extracts of cells transformed as those in FIG. **8**.

FIG. **10** shows a standard curve for ELISA assay of anti CEA activity.

FIGS. **11** and **12** show the construction of a plasmid for expression of the gene encoding a chimeric heavy chain.

FIG. **13** shows the construction of a plasmid for expression of the gene encoding the Fab region of heavy chain.

DETAILED DESCRIPTION

A. Definitions

As used herein, "antibodies" refers to tetramers or aggregates thereof which have specific immunoreactive activity, comprising light and heavy chains usually aggregated in the "Y" configuration of FIG. **1**, with or without covalent linkage between them; "immunoglobulins" refers to such assemblies whether or not specific immunoreactive activity is a property. "Non-specific immunoglobulin" ("NSI") means those immunoglobulins which do not possess specificity—i.e., those which are not antibodies.

"Mammalian antibodies" refers to antibodies wherein the amino acid sequences of the chains are homologous with those sequences found in antibodies produced by mammalian systems, either in situ, or in hybridomas. These antibodies mimic antibodies which are otherwise capable of being generated, although in impure form, in these traditional systems.

"Hybrid antibodies" refers to antibodies wherein chains are separately homologous with referenced mammalian antibody chains and represent novel assemblies of them, so that two different antigens are precipitable by the tetramer. In hybrid antibodies, one pair of heavy and light chain is homologous to antibodies raised against one antigen, while the other pair of heavy and light chain is homologous to those raised against another antigen. This results in the property of "divalence" i.e., ability to bind two antigens simultaneously. Such hybrids may, of course, also be formed using chimeric chains, as set forth below.

"Composite" immunoglobulins means those wherein the heavy and light chains mimic those of different species origins or specificities, and the resultant is thus likely to be a non-specific immunoglobulin (NSI), i.e. —lacking in antibody character.

"Chimeric antibodies" refers to those antibodies wherein one portion of each of the amino acid sequences of heavy and light chains is homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular class, while the remaining segment of the chains is homologous to corresponding sequences in another. Typically, in these chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals, while the constant portions are homologous to the sequences in antibodies derived from another. One clear advantage to such chimeric forms is that, for example, the variable regions can conveniently be derived from presently known sources using readily available hybridomas or B cells from non human host organisms in combination with constant regions derived from, for example, human cell preparations. While the variable region has the advantage of ease of preparation, and the specificity is not affected by its source, the constant region being human, is less likely to elicit an immune response from a human subject when the antibodies are injected than would the constant region from a non-human source.

However, the definition is not limited to this particular example. It includes any antibody in which either or both of the heavy or light chains are composed of combinations of sequences mimicking the sequences in antibodies of different sources, whether these sources be differing classes, differing antigen responses, or differing species of origin and whether or not the fusion point is at the variable/constant boundary. Thus, it is possible to produce antibodies in which neither the constant nor the variable region mimic known antibody sequences. It then becomes possible, for example, to construct antibodies whose variable region has a higher specific affinity for a particular antigen, or whose constant region can elicit enhanced complement fixation or to make other improvements in properties possessed by a particular constant region.

"Altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a mammalian or

other vertebrate antibody. Because of the relevance of recombinant DNA techniques to this invention, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the constant region. Changes in the constant region will, in general, be made in order to improve the cellular process characteristics, such as complement fixation, interaction with membranes, and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics. The antibody can also be engineered so as to aid the specific delivery of a toxic agent according to the "magic bullet" concept. Alterations, can be made by standard recombinant techniques and also by oligonucleotide-directed mutagenesis techniques (Dalbadie-McFarland, et al *Proc. Natl. Acad. Sci.* (*USA*), 79:6409 (1982)).

"Univalent antibodies" refers to aggregations which comprise a heavy chain/light chain dimer bound to the Fc (or stem) region of a second heavy chain. Such antibodies are specific for antigen, but have the additional desirable property of targeting tissues with specific antigenic surfaces, without causing its antigenic effectiveness to be impaired—i.e., there is no antigenic modulation. This phenomenon and the property of univalent antibodies in this regard is set forth in Glennie, M. J., et al., *Nature,* 295: 712 (1982). Univalent antibodies have heretofore been formed by proteolysis.

"Fab" region refers to those portions of the chains which are roughly equivalent, or analogous, to the sequences which comprise the Y branch portions of the heavy chain and to the light chain in its entirety, and which collectively (in aggregates) have been shown to exhibit antibody activity. "Fab protein", which protein is one of the aspects of the invention, includes aggregates of one heavy and one light chain (commonly known as Fab'), as well as tetramers which correspond to the two branch segments of the antibody Y, (commonly known as $F(ab)_2$), whether any of the above are covalently or non-covalently aggregated, so long as the aggregation is capable of selectively reacting with a particular antigen or antigen family. Fab antibodies have, as have univalent ones, been formed heretofore by proteolysis, and share the property of not eliciting antigen modulation on target tissues. However, as they lack the "effector" Fc portion they cannot effect, for example, lysis of the target cell by macrophages.

"Fab protein" has similar subsets according to the definition of the present invention as does the general term "antibodies" or "immunoglobulins". Thus, "mammalian" Fab protein, "hybrid" Fab protein "chimeric" Fab and "altered" Fab protein are defined analogously to the corresponding definitions set forth in the previous paragraphs for the various types of antibodies.

Individual heavy or light chains may of course be "mammalian", "chimeric" or "altered" in accordance with the above. As will become apparent from the detailed description of the invention, it is possible, using the techniques disclosed to prepare other combinations of the four-peptide chain aggregates, besides those specifically defined, such as hybrid antibodies containing chimeric light and mammalian heavy chains, hybrid Fab proteins containing chimeric Fab proteins of heavy chains associated with mammalian light chains, and so forth.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, i.e., the coding sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. A useful, but not a necessary, element of an effective expression vector is a marker encoding sequence—i.e. a sequence encoding a protein which results in a phenotypic property (e.g. tetracycline resistance) of the cells containing the protein which permits those cells to be readily identified. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified contained DNA code is included in this term, as it is applied to the specified sequence. As at present, such vectors are frequently in the form of plasmids, thus "plasmid" and "expression vector" are often used interchangeably. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which may, from time to time become known in the art.

"Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a recombinant host cell is by virtue of this transformation, rather than in such lesser amounts, or more commonly, in such less than detectable amounts, as would be produced by the untransformed host.

In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of antibody from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

B. Host Cell Cultures and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In general, of course, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATTC No. 31537).
These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F^-, λ^-, prototrophic, ATTC No. 27325), bacilli such as *Bacillus subtilus*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., *Gene* 2: 95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al, *Nature,* 275: 615 (1978);

Itakura, et al, *Science,* 198: 1056 (1977); (Goeddel, et al *Nature* 281: 544 (1979)) and a tryptophan (trp) promoter system (Goeddel, et al, *Nucleic Acids Res.,* 8: 4057 (1980); EPO Appl Publ No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist, et al, *Cell* 20: 269 (1980)).

In addition to prokaryates, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, (Stinchcomb, et al, *Nature,* 282: 39 (1979); Kingsman et al, *Gene,* 7: 141 (1979); Tschemper, et al, *Gene,* 10: 157 (1980)) is commonly used. This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics,* 85: 12 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.,* 255: 2073 (1980)) or other glycolytic enzymes (Hess, et al, *J. Adv. Enzyme Reg.,* 7: 149 (1968); Holland, et al, *Biochemistry,* 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization (Holland, ibid.). Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However interest has been greatest in vertebrate cells, and propogation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers, et al, *Nature,* 273: 113 (1978)) incorporated herein by reference. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g. Polyoma, Adeno, VSV, BPV, etc.) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

It will be understood that this invention, although described herein in terms of a preferred embodiment, should not be construed as limited to those host cells, vectors and expression systems exemplified.

C. Methods Employed

C.1 Transformation

If cells without formidable cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology,* 52: 546 (1978). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F. N. et al *Proc. Natl. Acad. Sci.* (*USA*), 69: 2110 (1972).

C.2 Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. The methods employed are not dependent on the DNA source, or intended host.

Cleavage is performed by treating with restriction enzyme (or enyzmes) in suitable buffer. In general, about 1 μg plasmid or DNA fragments is used with about 1 unit of enzyme in about 20 μl of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about 1 hour at 37° C. are workable. After incubations, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation is treated for 15 minutes at 15° with 10 units of *E. coli* DNA Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel, D., et al, *Nucleic Acids Res.,* 8: 4057 (1980) incorporated herein by reference.

For ligation, approximately equimolar amounts of the desired components, suitably end tailored to provide correct

matching are treated with about 10 units T4 DNA ligase per 0.5 μg DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

In the examples described below correct ligations for plasmid construction are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with the ligation mixture. Successful transformants were selected by ampicillin or tetracycline resistance depending on the mode of plasmid construction. Plasmids from the transformants were then prepared, analyzed by restriction and/or sequenced by the method of Messing, et al, *Nucleic Acids Res.,* 9:309 (1981) or by the method of Maxam, et al, *Methods in Enzymology,* 65:499 (1980).

D. Outline of Procedures

D.1 Mammalian Antibodies

The first type of antibody which forms a part of this invention, and is prepared by the methods thereof, is "mammalian antibody"-one wherein the heavy and light chains mimic the amino acid sequences of an antibody otherwise produced by a mature mammalian B lymphocyte either in situ or when fused with an immortalized cell as part of a hybridoma culture. In outline, these antibodies are produced as follows:

Messenger RNA coding for heavy or light chain is isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation, and the use of oligo-dT cellulose chromatography to segregate the poly-A mRNA. The poly-A mRNA may, further, be fractionated to obtain sequences of sufficient size to code for the amino acid sequences in the light or heavy chain of the desired antibody as the case may be.

A cDNA library is then prepared from the mixture of mRNA using a suitable primer, preferably a nucleic acid sequence which is characteristic of the desired cDNA. Such a primer may be hypothesized and synthesized based on the amino acid sequence of the antibody if the sequence is known. In the alternative cDNA from unfractionated poly-A mRNA from a cell line producing the desired antibody or poly-dT may also be used. The resulting cDNA is optionally size fractionated on polyacrylamide gel and then extended with, for example, dC residues for annealing with pBR322 or other suitable cloning vector which has been cleaved by a suitable restriction enzyme, such as Pst I, and extended with dG residues. Alternative means of forming cloning vectors containing the cDNA using other tails and other cloning vector remainder may, of course, also be used but the foregoing is a standard and preferable choice. A suitable host cell strain, typically *E. coli*, is transformed with the annealed cloning vectors, and the successful transformants identified by means of, for example, tetracycline resistance or other phenotypic characteristic residing on the cloning vector plasmid.

Successful transformants are picked and transferred to microtiter dishes or other support for further growth and preservation. Nitrocellulose filter imprints of these growing cultures are then probed with suitable nucleotide sequences containing bases known to be complementary to desired sequences in the cDNA. Several types of probe may be used, preferably synthetic single stranded DNA sequences labeled by kinasing with ATP^{32}. The cells fixed to the nitrocellulose filter are lysed, the DNA denatured, and then fixed before reaction with kinased probe. Clones which successfully hybridize are detected by contact with a photoplate, then plasmids from the growing colonies isolated and sequenced by means known in the art to verify that the desired portions of the gene are present.

The desired gene fragments are excised and tailored to assure appropriate reading frame with the control segments when inserted into suitable expression vectors. Typically, nucleotides are added to the 5' end to include a start signal and a suitably positioned restriction endonuclease site.

The tailored gene sequence is then positioned in a vector which contains a promoter in reading frame with the gene and compatible with the proposed host cell. A number of plasmids such as those described in U.S. Pat. Nos. 307,473; 291,892; and 305,657, have been described which already contain the appropriate promoters, control sequences, ribosome binding sites, and transcription termination sites, as well as convenient markers.

In the present invention, the gene coding for the light chain and that coding for the heavy chain are recovered separately by the procedures outlined above. Thus they may be inserted into separate expression plasmids, or together in the same plasmid, so long as each is under suitable promoter and translation control.

The expression vectors constructed above are then used to transform suitable cells. The light and heavy chains may be transformed into separate cell cultures, either of the same or of differing species; separate plasmids for light and heavy chain may be used to co-transform a single cell culture, or, finally, a single expression plasmid containing both genes and capable of expressing the genes for both light and heavy chain may be transformed into a single cell culture.

Regardless of which of the three foregoing options is chosen, the cells are grown under conditions appropriate to the production of the desired protein. Such conditions are primarily mandated by the type of promoter and control systems used in the expression vector, rather than by the nature of the desired protein. The protein thus produced is then recovered from the cell culture by methods known in the art, but choice of which is necessarily dependent on the form in which the protein is expressed. For example, it is common for mature heterologous proteins expressed in *E. coli* to be deposited within the cells as insoluble particles which require cell lysis and solubilization in denaturant to permit recovery. On the other hand, proteins under proper synthesis circumstances, in yeast and bacterial strains, can be secreted into the medium (yeast and gram positive bacteria) or into the periplasmic space (gram negative bacteria) allowing recovery by less drastic procedures. Tissue culture cells as hosts also appear, in general, to permit reasonably facile recovery of heterologous proteins.

When heavy and light chain are coexpressed in the same host, the isolation procedure is designed so as to recover reconstituted antibody. This can be accomplished in vitro as described below, or might be possible in vivo in a microorganism which secretes the IgG chains out of the reducing environment of the cytoplasm. A more detailed description is given in D.2, below.

D.2 Chain Recombination Techniques

The ability of the method of the invention to produce heavy and light chains or portions thereof, in isolation from each other offers the opportunity to obtain unique and unprecedented assemblies of immunoglobulins, Fab regions, and univalent antibodies. Such preparations require the use of techniques to reassemble isolated chains. Such means are known in the art, and it is, thus, appropriate to review them here.

While single chain disulfide bond containing proteins have been reduced and reoxidized to regenerate in high yield native structure and activity (Freedman, R. B., et al. In *Enzymology*

of Post Translational Modification of Proteins, I: 157-212 (1980) Academic Press, NY.), proteins which consist of discontinuous polypeptide chains held together by disulfide bonds are more difficult to reconstruct in vitro after reductive cleavage. Insulin, a cameo case, has received much experimental attention over the years, and can now be reconstructed so efficiently that an industrial process has been built around it (Chance, R. E., et al., In *Peptides: Proceedings of the Seventh Annual American Peptide Symposium* (Rich, D. H. and Gross, E., eds.) 721-728, Pierce Chemical Co., Rockford, Ill. (1981)).

Immunoglobulin has proved a more difficult problem than insulin. The tetramer is stabilized intra and intermolecularly by, 15 or more disulfide bonds. It has been possible to recombine heavy and light chains, disrupted by cleavage of only the interchain disulfides, to regain antibody activity even without restoration of the inter-chain disulfides (Edelman, G. M., et al., *Proc. Natl. Acad. Sci. (USA)* 50: 753 (1963)). In addition, active fragments of IgG formed by proteolysis (Fab fragments of ~50,000 MW) can be split into their fully reduced heavy chain and light chain components and fairly efficiently reconstructed to give active antibody (Haber, E., *Proc. Natl. Acad. Sci. (USA)* 52: 1099 (1964); Whitney, P. L., et al., *Proc. Natl. Acad. Sci. (USA)* 53: 524 (1965)). Attempts to reconstitute active antibody from fully reduced native IgG have been largely unsuccessful, presumably due to insolubility of the reduced chains and of side products or intermediates in the refolding pathway (see discussion in Freedman, M. H., et al., *J. Biol. Chem.* 241: 5225 (1966)). If, however, the immunoglobulin is randomly modified by polyalanylation of its lysines before complete reduction, the separated chains have the ability to recover antigen-combining activity upon reoxidation (ibid).

A particularly suitable method for immunoglobulin reconstitution is derivable from the now classical insulin recombination studies, wherein starting material was prepared by oxidative sulfitolysis, thus generating thiol-labile S-sulfonate groups at all cysteines in the protein, non-reductively breaking disulfides (Chance et al. (supra)). Oxidative sulfitolysis is a mild disulfide cleavage reaction (Means, G. E., et al., *Chemical Modification of Proteins*, Holden-Day, San Francisco (1971)) which is sometimes more gentle than reduction (Wetzel, R., *Biochemistry*, submitted (1983)), and which generates derivatives which are stable until exposed to mild reducing agent at which time disulfide reformation can occur via thiol-disulfide interchange (Morehead, H., et al. *Biochemistry*, in press, (1983)). In the present invention the heavy and light chain S-sulfonates generated by oxidative sulfitolysis were reconstituted utilizing both air oxidation and thiol-disulfide interchange to drive disulfide bond formation. The general procedure is set forth in detail in U.S. Pat. No. 452, 187, filed Dec. 22, 1982, incorporated herein by reference.

D.3 Variants Permitted by Recombinant Technology

Using the techniques described in paragraphs D.1 and D.2, additional operations which were utilized to gain efficient production of mammalian antibody can be varied in quite straightforward and simple ways to produce a great variety of modifications of this basic antibody form. These variations are inherent in the use of recombinant technology, which permits modification at a genetic level of amino acid sequences in normally encountered mammalian immunoglobulin chains, and the great power of this approach lies in its ability to achieve these variations, as well as in its potential for economic and specific production of desired scarce, and often contaminated, molecules. The variations also inhere in the ability to isolate production of individual chains, and thus create novel assemblies.

Briefly, since genetic manipulations permit reconstruction of genomic material in the process of construction of expression vectors, such reconstruction can be manipulated to produce new coding sequences for the components of "natural" antibodies or immunoglobulins. As discussed in further detail below, the coding sequence for a mammalian heavy chain may not be derived entirely from a single source or single species, but portions of a sequence can be recovered by the techniques described in D.1 from differing pools of mRNA, such as murine-murine hybridomas, human-murine hybridomas, or B cells differentiated in response to a series of antigen challenges. The desired portions of the sequences in each case can be recovered using the probe and analysis techniques described in D.1, and recombined in an expression vector using the same ligation procedures as would be employed for portions of the same model sequence. Such chimeric chains can be constructed of any desired length; hence, for example, a complete heavy chain can be constructed, or only sequence for the Fab region thereof.

The additional area of flexibility which arises from the use of recombinant techniques results from the power to produce heavy and light chains or fragments thereof in separate cultures or of unique combinations of heavy and light chain in the same culture, and to prevent reconstitution of the antibody or immunoglobulin aggregation until the suitable components are assembled. Thus, while normal antibody production results automatically in the formation of "mammalian antibodies" because the light and heavy chain portions are constructed in response to a particular determinant in the same cell, the methods of the present invention present the opportunity to assemble entirely new mixtures. Somewhat limited quantities of "hybrid" antibodies have been produced by "quadromas" i.e., fusions of two hybridoma cell cultures which permit random assemblies of the heavy and light chains so produced.

The present invention permits a more controlled assembly of desired chains, either by mixing the desired chains in vitro, or by transforming the same culture with the coding sequences for the desired chains.

D.4 Composite Immunoglobulins

The foregoing procedure, which describes in detail the recombinant production of mammalian antibodies is employed with some modifications to construct the remaining types of antibodies or NSIs encompassed by the present invention. To prepare the particular embodiment of composite non-specific immunoglobulin wherein the homology of the chains corresponds to the sequences of immunoglobulins of different specificities, it is of course, only necessary to prepare the heavy and light chains in separate cultures and reassemble them as desired.

For example, in order to make an anti-CEA light chain/anti-hepatitis heavy chain composite antibody, a suitable source for the mRNA used as a template for the light chain clone would comprise, for instance, the anti CEA producing cell line of paragraph E.1. The mRNA corresponding to heavy chain would be derived from B cells raised in response to hepatitis infection or from hybridoma in which the B cell was of this origin. It is clear that such composites can be assembled using the methods of the invention almost at will, and are limited only by available sources of mRNA suitable

for use as templates for the respective chains. All other features of the process are similar to those described above.

D.5 Hybrid Antibodies

Hybrid antibodies are particularly useful as they are capable of simultaneous reaction with more than one antigen. Pairs of heavy and light chains corresponding to chains of antibodies for different antigens, such as those set forth in paragraph D.4 are prepared in four separate cultures, thus preventing premature assembly of the tetramer. Subsequent mixing of the four separately prepared peptides then permits assembly into the desired tetramers. While random aggregation may lead to the formation of considerable undesired product, that portion of the product in which homologous light and heavy chains are bound to each other and mismatched to another pair gives the desired hybrid antibody.

D.6 Chimeric Antibodies

For construction of chimeric antibodies (wherein, for example, the variable sequences are separately derived from the constant sequences) the procedures of paragraph D.1 and D.2 are again applicable with appropriate additions and modifications. A preferred procedure is to recover desired portions of the genes encoding for parts of the heavy and light chains from suitable, differing, sources and then to religate these fragments using restriction endonucleases to reconstruct the gene coding for each chain.

For example, in a particularly preferred chimeric construction, portions of the heavy chain gene and of the light chain gene which encode the variable sequences of antibodies produced by a murine hybridoma culture are recovered and cloned from this culture and gene fragments encoding the constant regions of the heavy and light chains for human antibodies recovered and cloned from, for example, human myeloma cells. Suitable restriction enzymes may then be used to ligate the variable portions of the mouse gene to the constant regions of the human gene for each of the two chains. The chimeric chains are produced as set forth in D.1, aggregated as set forth in D.2 and used in the same manner as the non-chimeric forms. Of course, any splice point in the chains can be chosen.

D.7 Altered Antibodies

Altered antibodies present, in essence, an extension of chimeric ones. Again, the techniques of D.1 and D.2 are applicable; however, rather than splicing portions of the chain(s), suitable amino acid alterations, deletions or additions are made using available techniques such as mutagenesis (supra). For example, genes which encode antibodies having diminished complement fixation properties, or which have enhanced metal binding capacities are prepared using such techniques. The latter type may, for example, take advantage of the known gene sequence encoding metalothionein II (Karin, M., et al., *Nature,* 299: 797 (1982)). The chelating properties of this molecular fragment are useful in carrying heavy metals to tumor sites as an aid in tumor imaging (Scheinberg, D. A., et al., *Science,* 215: 19 (1982).

D.8 Univalent Antibodies

In another preferred embodiment, antibodies are formed which comprise one heavy and light chain pair coupled with the Fc region of a third (heavy) chain. These antibodies have a particularly useful property. They can, like ordinary antibodies, be used to target antigenic surfaces of tissues, such as tumors, but, unlike ordinary antibodies, they do not cause the antigenic surfaces of the target tissue to retreat and become non-receptive. Ordinary antibody use results in aggregation and subsequent inactivation, for several hours, of such surface antigens.

The method of construction of univalent antibodies is a straightforward application of the invention. The gene for heavy chain of the desired Fc region is cleaved by restriction enzymes, and only that portion coding for the desired Fc region expressed. This portion is then bound using the technique of D.2 to separately produced heavy chain the desired pairs separated from heavy/heavy and Fc/Fc combinations, and separately produced light chain added. Pre-binding of the two heavy chain portions thus diminishes the probability of formation of ordinary antibody.

D.9 Fab Protein

Similarly, it is not necessary to include the entire gene for the heavy chain portion. All of the aforementioned variations can be superimposed on a procedure for Fab protein production and the overall procedure differs only in that that portion of the heavy chain coding for the amino terminal 220 amino acids is employed in the appropriate expression vector.

E. Specific Examples of Preferred Embodiments

The invention has been described above in general terms and there follow several specific examples of embodiments which set forth details of experimental procedure in producing the desired antibodies. Example E.1 sets forth the general procedure for preparing anti CEA antibody components, i.e. for a "mammalian antibody". Example E.3 sets forth the procedure for reconstitution and thus is applicable to preparation of mammalian, composite, hybrid and chimeric immunoglobulins, and Fab proteins and univalent antibodies. Example E.4 sets forth the procedure for tailoring the heavy or light chain so that the variable and constant regions may be derived from different sources. Example E.5 sets forth the method of obtaining a shortened heavy chain genome which permits the production of the Fab regions and, in an analogous manner, Fc region.

The examples set forth below are included for illustrative purposes and do not limit the scope of the invention.

E.1 Construction of Expression Vectors for Murine Anti-CEA Antibody Chains and Peptide Synthesis

Carcinoembryonic antigen (CEA) is associated with the surface of certain tumor cells of human origin (Gold, P., et al., *J. Exp. Med.,* 122: 467 (1965)). Antibodies which bind to CEA (anti-CEA antibodies) are useful in early detection of these tumors (Van Nagell, T. R., et al., *Cancer Res.* 40: 502 (1980)), and have the potential for use in treatment of those human tumors which appear to support CEA at their surfaces. A mouse hybridoma cell line which secretes anti-CEA antibodies of the $Ig\gamma_1$ class, CEA.66-E3, has been prepared as described by Wagener, C, et al., *J. Immunol.* (in press) which is incorporated herein by reference, and was used as mRNA source. The production of anti CEA antibodies by this cell line was determined. The N-terminal sequences of the antibodies produced by these cells was compared with those of monoclonal anti CEA as follows. Purified IgG was treated with PCAse (Podell, D. N., et al., BBRC 81: 176 (1978)), and then dissociated in 6M guanidine hydrochloride, 10 mM 2-mercaptoethanol (1.0 mg of immunoglobulin, 5 min, 100° C. water bath). The dissociated chains were separated on a Waters Associates alkyl phenyl column using a linear gradi-

ent from 100 percent A (0.1 percent TFA-water) to 90 percent B (TFA/H_2O/MeCN 0.1/9.9/90) at a flow rate of 0.8 ml/min. Three major peaks were eluted and analyzed on SOS gels by silver staining. The first two peaks were pure light chain (MW 25,000 daltons), the third peak showed a (7:3) mixture of heavy and light chain. 1.2 nmoles of light chain were sequenced by the method of Shively, J. E., *Methods in Enzymology,* 79: 31 (1981), with an NH_2-terminal yield of 0.4 nmoles. A mixture of heavy and light chains (3 nmoles) was also sequenced, and sequence of light chain was deducted from the double sequence to yield the sequence of the heavy chain.

In the description which follows, isolation and expression of the genes for the heavy and light chains for anti CEA antibody produced by CEA.66-E3 are described. As the constant regions of these chains belong to the gamma and kappa families, respectively, "light chain" and "kappa chain", and "heavy chain" and "gamma chain", respectively, are used interchangeably below.

E.1.1 Isolation of Messenger RNA for Anti CEA Light and Heavy (Kappa and Gamma) Chains

Total RNA from CEA.66-E3 cells was extracted essentially as reported by Lynch et al, *Virology,* 98: 251 (1979). Cells were pelleted by centrifugation and approximately 1 g portions of pellet resuspended in 10 ml of 10 mM NaCl, 10 mM Tris HCl (pH 7.4), 1.5 mM $MgCl_2$. The resuspended cells were lysed by addition of non-ionic detergent NP-40 to a final concentration of 1 percent, and nuclei removed by centrifugation. After addition of SDS (pH 7.4) to 1 percent final concentration, the supernatant was extracted twice with 3 ml portions of phenol (redistilled)/chloroform:isoamyl alcohol 25:1 at 4° C. The aqueous phase was made 0.2 M in NaCl and total RNA was precipitated by addition of two volumes of 100 percent ethanol and overnight storage at −20° C. After centrifugation, polyA mRNA was purified from total RNA by oligo-dT cellulose chromatography as described by Aviv and Leder, *Proc. Nat'l. Acad. Sci.* (*USA*), 69: 1408 (1972). 142 μg of polyA mRNA was obtained from 1 g cells.

E.1.2 Preparation of *E. coli* Colony Library Containing Plasmids with Heavy and Light DNA Sequence Inserts

5 μg of the unfractionated polyA mRNA prepared in paragraph E.1.1 was used as template for oligo-dT primed preparation of double-stranded (ds) cDNA by standard procedures as described by Goeddel et al., *Nature* 281: 544 (1979) and Wickens et al., *J. Biol. Chem.* 253: 2483 (1978) incorporated herein by reference. The cDNA was size fractionated by 6 percent polyacrylamide gel electrophoresis and 124 ng of ds cDNA greater than 600 base pairs in length was recovered by electroelution. A 20 ng portion of ds cDNA was extended with deoxy C residues using terminal deoxynucleotidyl transferase as described in Chang et al., *Nature* 275: 617 (1978) incorporated herein by reference, and annealed with 200 ng of the plasmid pBR322 (Bolivar et al., *Gene* 2: 95 (1977)) which had been cleaved with Pst I and tailed with deoxy G. Each annealed mixture was then transformed into *E. coli* K12 strain 294 (ATCC No. 31446). Approximately 8500 ampicillin sensitive, tetracycline resistant transformants were obtained.

E.1.3 Preparation of Synthetic Probes

The 14mer, 5' GGTGGGAAGATGGA 3' complementary to the coding sequence of constant region for mouse MOPC21 kappa chain which begins 25 basepairs 3' of the variable region DNA sequence was used as kappa chain probe. A 15 mer, 5' GACCAGGCATCCCAG 3', complementary to a coding sequence located 72 basepairs 3' of the variable region DNA sequence for mouse MOPC21 gamma chain was used to probe gamma chain gene.

Both probes were synthesized by the phosphotriester method described in German Offenlegungschrift 2644432, incorporated herein by reference, and made radioactive by kinasing as follows: 250 ng of deoxyoligonucleotide were combined in 25 μl of 60 mM Tris HCl (pH 8), 10 mM $MgCl_2$, 15 mM beta-mercaptoethanol, and 100 μCi (γ-^{32}P) ATP (Amersham, 5000 Ci/mMole). 5 units of T4 polynucleotide kinase were added and the reaction was allowed to proceed at 37° C. for 30 minutes and terminated by addition of EDTA to 20 mM.

E.1.4 Screening of Colony Library for Kappa or Gamma Chain Sequences

~2000 colonies prepared as described in paragraph E.1.2 were individually inoculated into wells of microtitre dishes containing LB (Miller, Experiments in Molecular Genetics, p. 431-3, Cold Spring Harbor Lab., Cold Spring Harbor, N.Y. (1972))+5 μg/ml tetracycline and stored at −20° C. after addition of DMSO to 7 percent. Individual colonies from this library were transferred to duplicate sets of Schleicher and Schuell BA85/20 nitrocellulose filters and grown on agar plates containing LB+5 μg/ml tetracycline. After ~10 hours growth at 37° C. the colony filters were transferred to agar plates containing LB+5 μg/ml tetracycline and 12.5 μg/ml chloramphenicol and reincubated overnight at 37° C. The DNA from each colony was then denatured and fixed to the filter by a modification of the Grunstein-Hogness procedure as described in Grunstein et al., *Proc. Natl. Acad. Sci.* (*USA*) 72: 3961 (1975), incorporated herein by reference. Each filter was floated for 3 minutes on 0.5 N NaOH, 1.5 M NaCl to lyse the colonies and denature the DNA then neutralized by floating for 15 minutes on 3 M NaCl, 0.5 M Tris HCl (pH 7.5). The filters were then floated for an additional 15 minutes on 2×SSC, and subsequently baked for 2 hours in an 80° C. vacuum oven. The filters were prehybridized for ~2 hours at room temperature in 0.9 M NaCl, 1×Denhardts, 100 mM Tris HCl (pH 7.5), 5 mM Na-EDTA, 1 mM ATP, 1 M sodium phosphate (dibasic), 1 mM sodium pyrophosphate, 0.5 percent NP-40, and 200 μg/ml *E. coli* t-RNA, and hybridized in the same solution overnight, essentially as described by Wallace et al. *Nucleic Acids Research* 9: 879 (1981) using ~40× 10^6 cpm of either the kinased kappa or gamma probe described above.

After extensive washing at 37° C. in 6×SSC, 0.1 percent SDS, the filters were exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens for 16-24 hours at −80° C. Approximately 20 colonies which hybridized with kappa chain probe and 20 which hybridized with gamma chain probe were characterized.

E.1.5 Characterization of Colonies which Hybridize to Kappa DNA Sequence Probe

Plasmid DNAs isolated from several different transformants which hybridized to kappa chain probe were cleaved with Pst I and fractionated by polyacrylamide gel electrophoresis (PAGE). This analysis demonstrated that a number of plasmid DNAs contained cDNA inserts large enough to encode full length kappa chain. The complete nucleotide sequence of the cDNA insert of one of these plasmids was determined by the dideoxynucleotide chain termination method as described by Smith, *Methods Enzymol.* 65, 560 (1980) incorporated herein by reference after subcloning restriction endonuclease cleavage fragments into M13 vectors (Messing et al., *Nucleic Acids Research* 9: 309 (1981). FIG. **2** shows the nucleotide sequence of the cDNA insert of pK17G4 and FIG. **3** shows the gene sequence with the corresponding amino acid sequence. Thus, the entire coding region of mouse anti-CEA kappa chain was isolated on this one large DNA fragment. The amino acid sequence of kappa chain,

deduced from the nucleotide sequence of the pK17G4 cDNA insert, corresponds perfectly with the first 23 N-terminal amino acids of mature mouse anti-CEA kappa chain as determined by amino acid sequence analysis of purified mouse anti-CEA kappa chain. The coding region of pK17G4 contains 27 basepairs or 9 amino acids of the presequence and 642 basepairs or 214 amino acids of the mature protein. The mature unglycosylated protein (MW 24,553) has a variable region of 119 amino acids, including the J1 joining region of 12 amino acids, and a constant region of 107 amino acids. After the stop codon behind amino acid 215 begins 212 basepairs of 3' untranslated sequence up to the polyA addition. The kappa chain probe used to identify pK17G4 hybridizes to nucleotides 374-388 (FIG. **2**).

E.1.6 Characterization of Colonies which Hybridize to Gamma 1 DNA Probe

Plasmid DNA isolated from several transformants positive for hybridization with the heavy chain gamma 1 probe was subjected to Pst I restriction endonuclease analysis as described in E.1.5. Plasmid DNAs demonstrating the largest cDNA insert fragments were selected for further study. Nucleotide sequence coding for mouse heavy (gamma-1) chain, shows an NcoI restriction endonuclease cleavage site near the junction between variable and constant region. Selected plasmid DNAs were digested with both PstI and NcoI and sized on polyacrylamide. This analysis allowed identification of a number of plasmid DNAs that contain NcoI restriction endonuclease sites, although none that demonstrate cDNA insert fragments large enough to encode the entire coding region of mouse anti-CEA heavy chain.

In one plasmid isolated, p γ298 the cDNA insert of about 1300 bp contains sequence information for the 5' untranslated region, the signal sequence and the N-terminal portion of heavy chain. Because pγ298 did not encode the C-terminal sequence for mouse anti-CEA gamma 1 chain, plasmid DNA was isolated from other colonies and screened with PstI and NcoI. The C-terminal region of the cDNA insert of pγ11 was sequenced and shown to contain the stop codon, 3' untranslated sequence and that portion of the coding sequence missing from p γ298.

FIG. **4** presents the entire nucleotide sequence of mouse anti-CEA heavy chain (as determined by the dideoxynucleotide chain termination method of Smith, *Methods Enzymol.*, 65: 560 (1980)) and FIG. **5** includes the translated sequence.

The amino acid sequence of gamma 1 (heavy chain) deduced from the nucleotide sequence of the pγ298 cDNA insert corresponds perfectly to the first 23 N-terminal amino acids of mature mouse anti-CEA gamma 1 chain as determined by amino acid sequence analysis of purified mouse anti-CEA gamma-1 chain. The coding region consists of 57 basepairs or 19 amino acids of presequences and 1346 basepairs or 447 amino acids of mature protein. The mature unglycosolated protein (MW 52,258) has a variable region of 135 amino acids, including a D region of 12 amino acids, and a J4 joining region of 13 amino acids. The constant region is 324 amino acids. After the stop codon behind amino acid 447 begins 96 bp of 3' untranslated sequences up to the polyA addition. The probe used to identify pγ298 and pγ11 hybridized to nucleotides 528-542 (FIG. **4**).

E.1.7 Construction of a Plasmid for Direct Expression of Mouse Mature Anti-CEA Kappa Chain Gene, pKCE-Atrp207-1☆

FIG. **6** illustrates the construction of pKCEAtrp207-1☆

First, an intermediate plasmid pHGH207-1☆, having a single trp promoter, was prepared as follows:

The plasmid pHGH 207 (described in U.S. Pat. No. 307, 473, filed Oct. 1, 1981) has a double lac promoter followed by the trp promoter, flanked by EcoR I sites and was used to prepare pHGH207-1. pHGH207 was digested with BamH 1, followed by partial digestion with EcoR I. The largest fragment, which contains the entire trp promoter, was isolated and ligated to the largest EcoR I-BamH I fragment from pBR322, and the ligation mixture used to transform *E. coli* 294. Tet^R Amp^R colonies were isolated, and most of them contained pHGH207-1. pHGH207-1☆ which lacks the EcoR1 site between the amp^R gene and the trp promoter, was obtained by partial digestion of pHGH207-1 with EcoR I, filling in the ends with Klenow and dNTPs, and religation.

5 μg of pHGH207-1☆ was digested with EcoRI, and the ends extended to blunt ends using 12 units of DNA Polymerase I in a 50 μl reaction containing 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4) and 1 mM in each dNTP at 37° C. for 1 hour, followed by extraction with phenol/$CHCl_3$ and precipitation with ethanol. The precipitated DNA was digested with BamH I, and the large vector fragment (fragment 1) purified using 5 percent polyacrylamide gel electrophoresis, electroelution, phenol/$CHCl_3$ extraction and ethanol precipitation.

The DNA was resuspended in 50 μl of 10 mM Tris pH 8, 1 mM EDTA and treated with 500 units Bacterial Alkaline Phosphatase (BAP) for 30' at 65° followed by phenol/$CHCl_3$ extraction and ethanol precipitation.

A DNA fragment containing part of the light chain sequence was prepared as follows: 7 μg of pK17G4 DNA was digested with Pst I and the kappa chain containing cDNA insert was isolated by 6 percent gel electrophoresis, and electroelution. After phenol/$CHCl_3$ extraction, ethanol precipitation and resuspension in water, this fragment was digested with Ava II. The 333 bp Pst I-Ava II DNA fragment was isolated and purified from a 6 percent polyacrylamide gel.

A 15 nucleotide DNA primer was synthesized by the phosphotriester method G. O. 2,644,432 (supra) and has the following sequence:

```
Met Asp Ile Val Met
5' ATG GAC ATT GTT ATG 3'
```

The 5' methionine serves as the initiation codon. 500 ng of this primer was phosphorylated at the 5' end with 10 units T4 DNA kinase in 20 μl reaction containing 0.5 mM ATP. ~200 ng of the Pst I-Ava II DNA fragment was mixed with the 20 μl of the phosphorylated primer, heated to 95° C. for 3 minutes and quick frozen in a dry-ice ethanol bath. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C. this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with Sau 3A. The reaction mixture was then electrophoresed on a 6 percent polyacrylamide gel and ~50 ng of the 182 basepair amino-terminal blunt-end to Sau 3A fragment (fragment 2) was obtained after electroelution.

100 ng of fragment 1 (supra) and 50 ng of fragment 2 were combined in 20 μl of 20 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 2.5 mM ATP and 1 unit of 14 DNA ligase. After overnight ligation at 14° C. the reaction was transformed into *E. coli* K12 strain 294. Restriction endonuclease digestion of plasmid DNA from a number of ampicillin resistant transformants indicated the proper construction and DNA sequence analysis proved the desired nucleotide sequence through the initiation codon of this new plasmid, pKCEAInt1 (FIG. **6**).

The remainder of the coding sequence of the kappa light chain gene was prepared as follows:

The Pst I cDNA insert fragment from 7 μg of K17G4 DNA was partially digested with Ava II and the Ava II cohesive ends were extended to blunt ends in a DNA Polymerase I large fragment reaction. Following 6 percent polyacrylamide gel electrophoresis the 686 basepair Pst I to blunt ended Ava II DNA fragment was isolated, purified and subjected to Hpa II restriction endonuclease digestion. The 497 basepair Hpa II to blunt ended Ava II DNA fragment (fragment 3) was isolated and purified after gel electrophoresis.

10 μg of pKCEAInt1 DNA was digested with Ava I, extended with DNA polymerase I large fragment, and digested with Xba I. Both the large blunt ended Ava I to Xba I vector fragment and the small blunt ended Ava I to Xba I fragment were isolated and purified from a 6 percent polyacrylamide gel after electrophoresis. The large vector fragment (fragment 4) was treated with Bacterial Alkaline Phosphatase (BAP), and the small fragment was digested with Hpa II, electrophoresed on a 6 percent polyacrylamide and the 169 basepair Xba I-Hpa II DNA fragment (fragment 5) was purified. ~75 ng of fragment 4, ~50 ng of fragment 3 and ~50 ng of fragment 5 were combined in a T4 DNA ligase reaction and incubated overnight at 14°, and the reaction mixture transformed into *E. coli* K12 strain 294. Plasmid DNA from six ampicillin resistant transformants were analyzed by restriction endonuclease digestion. One plasmid DNA demonstrated the proper construction and was designated pKCEAInt2.

Final construction was effected by ligating the K-CEA fragment, including the trp promoter from pKCEAInt2 into pBR322(XAP). (pBR322(XAP) is prepared as described in U.S. Pat. No. 452,227, filed Dec. 22, 1982.)

The K-CEA fragment was prepared by treating pKCEAInt2 with Ava I, blunt ending with DNA polymerase I (Klenow fragment) in the presence of DNTPs, digestion with Pst I and isolation of the desired fragment by gel electrophoresis and electroelution.

The large vector fragment from pBR322(XAP) was prepared by successive treatment with EcoR I, blunt ending with polymerase, and redigestion with Pst I, followed by isolation of the large vector fragment by electrophoresis and electroelution.

The K-CEA and large vector fragments as prepared in the preceding paragraphs were ligated with T4 DNA ligase, and the ligation mixture transformed into *E. coli* as above. Plasmid DNA from several ampicillin resistant transformants were selected for analysis, and one plasmid DNA demonstrated the proper construction, and was designated pKCEAtrp207-I☆.

E.1.8 Construction of a Plasmid Vector for Direct Expression of Mouse Mature Anti-CEA Heavy (Gamma 1) Chain Gene, pγCEAtrp207-1☆

FIG. **7** illustrates the construction of pγCEAtrp207-1☆. This plasmid was constructed in two parts beginning with construction of the C-terminal region of the gamma 1 gene.

5 μg of plasmid pHGH207-1☆ was digested with Ava I, extended to blunt ends with DNA polymerase I large fragment (Klenow fragment), extracted with phenol/$CHCl_3$, and ethanol precipitated. The DNA was digested with BamH I treated with BAP and the large fragment (fragment A) was purified by 6 percent polyacrylamide gel electrophoresis and electroelution.

~5 μg of pγ11 was digested with Pst I and the gamma chain cDNA insert fragment containing the C-terminal portion of the gene was purified, digested with Ava II followed by extension of the Ava II cohesive ends with Klenow, followed by Taq I digestion. The 375 basepair blunt ended Ava II to Taq I fragment (fragment B) was isolated and purified by gel electrophoresis and electroelution.

9 μg of pγ298 was digested with Taq I and BamH I for isolation of the 496 basepair fragment (fragment C).

Approximately equimolar amounts of fragments A, B, and C were ligated overnight at 14° in 20 μl reaction mixture, then transformed into *E. coli* strain 294. The plasmid DNA from six ampicillin resistant transformants was committed to restriction endonuclease analysis and one plasmid DNA, named pγCEAInt, demonstrated the correct construction of the C-terminal portion of gamma 1 (FIG. **5**).

To obtain the N-terminal sequences, 30 μg of pγ298 was digested with Pst I and the 628 basepair DNA fragment encoding the N-terminal region of mouse anti-CEA gamma chain was isolated and purified. This fragment was further digested with Alu I and Rsa I for isolation of the 280 basepair fragment. A 15 nucleotide DNA primer

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        met glu val met leu
5' ATG GAA GTG ATG CTG 3'
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was synthesized by the phosphotriester method (supra).

The 5' methionine serves as the initiation codon. 500 ng of this synthetic oligomer primer was phosphorylated at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl reaction mixture. ~500 ng of the 280 basepair Alu I-Rsa I DNA fragment was mixed with the phosphorylated primer. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with HpaII. ~50 ng of the expected 125 basepair blunt-end to Hpa II DNA fragment (fragment D) was purified from the gel.

A second aliquot of pγ298 DNA was digested with Pst I, the 628 basepair DNA fragment purified by polyacrylamide gel electrophoresis, and further digested with BamH I and Hpa II. The resulting 380 basepair fragment (fragment E) was purified by gel electrophoresis.

~5 μg of pγCEAIntI was digested with EcoR I, the cohesive ends were made flush with DNA polymerase I (Klenow), further digested with BamH I, treated with BAP and electrophoresed on a 6 percent polyacrylamide gel. The large vector fragment (fragment F) was isolated and purified.

In a three fragment ligation, 50 ng fragment D, 100 ng fragment E, and 100 ng fragment F were ligated overnight at 4° in a 20 μl reaction mixture and used to transform *E. coli* K12 strain 294. The plasmid DNAs from 12 ampicillin resistant transformants were analyzed for the correct construction and the nucleotide sequence surrounding the initiation codon was verified to be correct for the plasmid named pγCEAInt2.

The expression plasmid, pγCEAtrp207-I☆ used for expression of the heavy chain gene is prepared by a 3-way ligation using the large vector fragment from pBR322(XAP) (supra) and two fragments prepared from pγCEAInt2.

pBR322(XAP) was treated as above by digestion with EcoR1, blunt ending with DNA polymerase (Klenow) in the presence of dNTPs, followed by digestion with Pst I, and isolation of the large vector fragment by gel electrophoresis. A 1543 base pair fragment from pγCEAInt2 containing trp promoter linked with the N-terminal coding region of the heavy chain gene was isolated by treating pγCEAInt2 with Pst

I followed by BamH I, and isolation of the desired fragment using PAGE. The 869 base pair fragment containing the C-terminal coding portion of the gene was prepared by partial digestion of pγCEAInt2 with Ava I, blunt ending with Klenow, and subsequent digestion with BamH I, followed by purification of the desired fragment by gel electrophoresis.

The aforementioned three fragments were then ligated under standard conditions using T4 DNA ligase, and a ligation mixture used to transform *E. coli* strain 294. Plasmid DNAs from several tetracycline resistant transformants were analyzed; one plasmid DNA demonstrated the proper construction and was designated pγCEAtrp207-1☆.

E.1.9 Production of Immunoglobulin Chains by *E. coli*

E. coli strain W3110 (ATTC No. 27325) was transformed with pγCEAtrp207-1☆ or pKCEAtrp207-1☆ using standard techniques.

To obtain double transformants, *E. coli* strain W3110 cells were transformed with a modified pKCEAtrp207-1☆, pKCEAtrp207-1☆Δ, which had been modified by cleaving a Pst I-Pvu I fragment from the amp^R gene and religating. Cells transformed with pKCEAtrp207-1☆Δ are thus sensitive to ampicillin but still resistant to tetracycline. Successful transformants were retransformed using pγCEAInt2 which confers resistance to ampicillin but not tetracycline. Cells containing both pKCEAtrp207-1☆Δ and pγCEAInt2 thus identified by growth in a medium containing both ampicillin and tetracycline.

To confirm the production of heavy and/or light chains in the transformed cells, the cell samples were inoculated into M9 tryptophan free medium containing 10 μg/ml tetracycline, and induced with indoleacrylic acid (IAA) when the OD 550 reads 0.5. The induced cells were grown at 37° C. during various time periods and then spun down, and suspended in TE buffer containing 2 percent SDS and 0.1 M β-mercaptoethanol and boiled for 5 minutes. A 10× volume of acetone was added and the cells kept at 22° C. for 10 minutes, then centrifuged at 12,000 rpm. The precipitate was suspended in O'Farrell SDS sample buffer (O'Farrell, P. H., *J. Biol. Chem.*, 250: 4007 (1975)); boiled 3 minutes, recentrifuged, and fractionated using SDS PAGE (10 percent), and stained with silver stain (Goldman, D. et al., *Science* 211: 1437 (1981)); or subjected to Western blot using rabbit anti-mouse IgG (Burnett, W. N., et al., *Anal. Biochem.* 112: 195 (1981)), for identification light chain and heavy chain.

Cells transformed with pγCEAtrp207-1☆ showed bands upon SDS PAGE corresponding to heavy chain molecular weight as developed by silver stain. Cells transformed with pKCEAtrp207-1☆ showed the proper molecular weight band for light chain as identified by Western blot; double transformed cells showed bands for both heavy and light chain molecular weight proteins when developed using rabbit anti-mouse IgG by Western blot. These results are shown in FIGS. **8**A, **8**B, and **8**C.

FIG. **8**A shows results developed by silver stain from cells transformed with pγCEAtrp207-1☆. Lane 1 is monoclonal anti-CEA heavy chain (standard) from CEA.66-E3. Lanes 2b-5b are timed samples 2 hrs, 4 hrs, 6 hrs, and 24 hrs after IAA addition. Lanes 2a-5a are corresponding untransformed controls; Lanes 2c-5c are corresponding uninduced transformants.

FIG. **8**B shows results developed by Western blot from cells transformed with pKCEAtrp207-1☆. Lanes 1b-6b are extracts from induced cells immediately, 1 hr, 3.5 hrs, 5 hrs, 8 hrs, and 24 hrs after IAA addition, and 1a-6a corresponding uninduced controls. Lane 7 is an extract from a pγCEAtrp207-1☆ control, lanes 8, 9, and 10 are varying amounts of anti CEA-kappa chain from CEA.66-E3 cells.

FIG. **8**C shows results developed by Western blot from four colonies of double transformed cells 24 hours after IAA addition (lanes 4-7). Lanes 1-3 are varying amounts of monoclonal gamma chain controls, lanes 8 and 9 are untransformed and pγCEAtrp207-1☆ transformed cell extracts, respectively.

In another quantitative assay, frozen, transformed *E. coli* cells grown according to E.1.10 (below) were lysed by heating in sodium dodecyl sulfate (SDS)/γ-mercaptoethanol cell lysis buffer at 100°. Aliquots were loaded on an SDS polyacrylamide gel next to lanes loaded with various amounts of hybridoma anti-CEA. The gel was developed by the Western blot, Burnett (supra), using ^{125}I-labeled sheep anti-mouse IgG antibody from New England Nuclear. The results are shown in FIG. **9**. The figure shows that the *E. coli* products co-migrate with the authentic hybridoma chains, indicating no detectable proteolytic degradation in *E. coli*. Heavy chain from mammalian cells is expected to be slightly heavier than *E. coli* material due to glycosylation in the former. Using the hybridoma lanes as a standard, the following estimates of heavy and light chain production were made:

	(Per gram of cells)
E. coli (W3110/pγCE Atrp207-1*)	5 mg γ
E. coli (W3110/pKCE Atrp207-1*)	1.5 mg K
E. coli (W3110/pKCE Atrp207-1*Δ, pγCEAInt2)	0.5 mg K, 1.0 mg γ

E.1.10 Reconstitution of Antibody from Recombinant K and Gamma Chains

In order to obtain heavy and light chain preparations for reconstitution, transformed cells were grown in larger batches, harvested and frozen. Conditions of growth of the variously transformed cells were as follows:

E. coli (W3110/pγCEAtrp207-1☆) were inoculated into 500 ml LB medium containing 5 μg/ml tetracycline and grown on a rotary shaker for 8 hours. The culture was then transferred to 10 liters of fermentation medium containing yeast nutrients, salts, glucose, and 2 μg/ml tetracycline. Additional glucose was added during growth and at OD 550=20, indoleacrylic (IAA), a trp derepressor, was added to a concentration of 50 μg/ml. The cells were fed additional glucose to a final OD 550=40, achieved approximately 6 hours from the IAA addition.

E. coli (W3110) cells transformed with pKCEA trp 207-1☆ and double transformed (with pKCEAtrp207-1☆Δ and pγCEAInt2) were grown in a manner analogous to that described above except that the OD 550 six hours after IAA addition at harvest was 25-30.

The cells were then harvested by centrifugation, and frozen.

E.2 Assay Method for Reconstituted Antibody

Anti-CEA activity was determined by ELISA as a criterion for successful reconstitution. Wells of microtiter plates (Dynatech Immulon) were saturated with CEA by incubating 100 μl of 2-5 μg CEA/ml solution in 0.1M carbonate buffer, pH 9.3 for 12 hours at room temperature. The wells were then washed 4 times with phosphate buffered saline (PBS), and then saturated with BSA by incubating 200 μl of 0.5 percent BSA in PBS for 2 hours at 37° C., followed by washing 4 times with PBS. Fifty microliters of each sample was applied to each well. A standard curve (shown in FIG. **10**), was run, which consisted of 50 μl samples of 10 μg, 5 μg, 1 μg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng and 1 ng anti-CEA/ml in 0.5 percent BSA in PBS, plus 50 μl of 0.5 percent BSA in PBS alone as a blank. All of the samples were incubated in the plate for 90 minutes at 37° C.

The plates were then washed 4 times with PBS, and sheep anti-mouse IgG-alkaline phosphate (TAGO, Inc.) was applied to each well by adding 100 μl of an enzyme concentration of 24 units/ml in 0.5 percent BSA in PBS. The solution was incubated at 37° C. for 90 minutes. The plates were washed 4 times with PBS before adding the substrate, 100 μl of a 0.4 mg/ml solution of p-nitrophenylphosphate (Sigma) in ethanolamine buffered saline, pH 9.5. The substrate was incubated 90 minutes at 37° C. for color development.

The A_{450} of each well was read by the Microelisa Auto Reader (Dynatech) set to a threshold of 1.5, calibration of 1.0 and the 0.5 percent BSA in PBS (Blank) well set to 0.000. The A_{450} data was tabulated in RS-1 on the VAX system, and the standard curve data fitted to a four-parameter logistic model. The unknown samples' concentrations were calculated based on the A_{450} data.

E.3 Reconstitution of Recombinant Antibody and Assay

Frozen cells prepared as described in paragraph E.1.10 were thawed in cold lysis buffer [10 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication. The lysate was partially clarified by centrifugation for 20 mins at 3,000 rpm. The supernatant was protected from proteolytic enzymes by an additional 1 mM PMSF, and used immediately or stored frozen at −80° C.; frozen lysates were never thawed more than once.

The S-sulfonate of *E. coli* produced anti-CEA heavy chain (γ) was prepared as follows: Recombinant *E. coli* cells transformed with pγCEAtrp207-1☆ which contained heavy chain as insoluble bodies, were lysed and centrifuged as above; the pellet was resuspended in the same buffer, sonicated and re-centrifuged. This pellet was washed once with buffer, then suspended in 6M guanidine HCl, 0.1M Tris HCl, pH 8, 1 mM EDTA, 20 mg/ml sodium sulfite and 10 mg/ml sodium tetrathionate and allowed to react at 25° for about 16 hrs. The reaction mixture was dialyzed against 8M urea, 0.1M Tris HCl, pH 8, and stored at 4°, to give a 3 mg/ml solution of γ-SSO_3.

650 μl of cell lysate from cells of various *E. coli* strains producing various IgG chains, was added to 500 mg urea. To this was added β-mercaptoethanol to 20 mM, Tris-HCl, pH 8.5 to 50 mM and EDTA to 1 mM, and in some experiments, γ-SSO_3 was added to 0.1 mg/ml. After standing at 25° for 30-90 mins., the reaction mixtures were dialyzed at 4° against a buffer composed of 0.1M sodium glycinate, pH 10.8, 0.5M urea, 10 mM glycine ethyl ester, 5 mM reduced glutathione, 0.1 mM oxidized glutathione. This buffer was prepared from N_2-saturated water and the dialysis was performed in a capped Wheaton bottle. After 16-48 hours, dialysis bags were transferred to 4° phosphate buffered saline containing 1 mM PMSF and dialysis continued another 16-24 hrs. Dialysates were assayed by ELISA as described in paragraph E.2 for ability to bind CEA. The results below show the values obtained by comparison with the standard curve in×ng/ml anti-CEA. Also shown are the reconstitution efficiencies calculated from the ELISA responses, minus the background (108 ng/ml) of cells producing K chain only, and from estimates of the levels of γ and K chains in the reaction mixtures.

	ng/ml anti-CEA	Percent recombination
E. coli W3110 producing IFN-αA (control)	0	—
E. coli (W3110/pKCEAtrp207-1*)	108	—
E. coli (W3110/pKCEAtrp207-1*), plus γ-$SS0_3$	848	0.33
E. coli (W3110/pKCEAtrp207-1*Δ, pγCEAInt2)	1580	0.76
Hybridoma anti-CEA K-$SS0_3$ and γ-$SS0_3$	540	0.40

E.4 Preparation of Chimeric Antibody

FIGS. **11** and **12** show the construction of an expression vector for a chimeric heavy (gamma) chain which comprises the murine anti CEA variable region and human γ-2 constant region.

A DNA sequence encoding the human gamma-2 heavy chain is prepared as follows: the cDNA library obtained by standard techniques from a human multiple myeloma cell line is probed with 5' GGGCACTCGACACAA 3' to obtain the plasmid containing the cDNA insert for human gamma-2 chain (Takahashi, et al., *Cell,* 29: 671 (1982), incorporated herein by reference), and analyzed to verify its identity with the known sequence in human gamma-2 (Ellison, J., et al., *Proc. Natl. Acad. Sci.* (*USA*), 79: 1984 (1982) incorporated herein by reference).

As shown in FIG. **11**, two fragments are obtained from this cloned human gamma 2 plasmid (pγ2). The first fragment is formed by digestion with PvuII followed by digestion with Ava III, and purification of the smaller DNA fragment, which contains a portion of the constant region, using 6 percent PAGE. The second fragment is obtained by digesting the pγ2 with any restriction enzyme which cleaves in the 3' untranslated region of γ2, as deduced from the nucleotide sequence, filling in with Klenow and dNTPs, cleaving with Ava III, and isolating the smaller fragment using 6 percent PAGE. (The choice of a two step, two fragment composition to supply the PvuII-3' untranslated fragment provides a cleaner path to product due to the proximity of the AvaIII site to the 3 terminal end thus avoiding additional restriction sites in the gene sequence matching the 3' untranslated region site.) pγCEA207-1☆ is digested with EcoR 1, treated with Klenow and dNTPs to fill in the cohesive end, and digested with Pvu II, the large vector fragment containing promoter isolated by 6 percent PAGE.

The location and DNA sequence surrounding the PvuII site in the mouse gamma-1 gene are identical to the location and DNA sequence surrounding the PvuII site in the human gamma-2 gene.

The plasmid resulting from a three way ligation of the foregoing fragments, pChim1, contains, under the influence of trp promoter, the variable and part of the constant region of murine anti-CEA gamma 1 chain, and a portion of the gamma 2 human chain. pChim1 will, in fact, express a chimeric heavy chain when transformed into *E. coli*, but one wherein the change from mouse to human does not take place at the variable to constant junction.

FIG. **12** shows modification of pChim1 to construct pChim2 so that the resulting protein from expression will contain variable region from murine anti CEA antibody and constant region from the human γ-2 chain. First, a fragment is prepared from pChim1 by treating with Nco I, blunt ending with Klenow and dNTPs, cleaving with Pvu II, and isolating the large vector fragment which is almost the complete plasmid except for short segment in the constant coding region for mouse anti CEA. A second fragment is prepared from the previously described pγ2 by treating with Pvu II, followed by treating with any restriction enzyme which cleaves in the variable region, blunt ending with Klenow and dNTPs and

isolating the short fragment which comprises the junction between variable and constant regions of this chain.

Ligation of the foregoing two fragments produces an intermediate plasmid which is correct except for an extraneous DNA fragment which contains a small portion of the constant region of the murine anti CEA antigen, and a small portion of the variable region of the human gamma chain. This repair can be made by excising the Xba I to Pvu II fragment and cloning into M13 phage as described by Messing et al., *Nucleic Acids Res.* 9: 309 (1981), followed by in vitro site directed deletion mutagenesis as described by Adelman, et al., *DNA,* in press (1983) which is incorporated herein by reference. The Xba I-Pvu II fragment thus modified is ligated back into the intermediate plasmid to form pChim2. This plasmid then is capable of expressing in a suitable host a cleanly constructed murine variable/human constant chimeric heavy chain.

In an analogous fashion, but using mRNA templates for cDNA construction for human kappa rather than γ chain, the expression plasmid for chimeric light chain is prepared.

The foregoing two plasmids are then double transformed into *E. coli* W3110, the cells grown and the chains reconstituted as set forth in paragraph E.1-E.3 supra;

E.5 Preparation of Altered Murine Anti-CEA Antibody

E.5.1 Construction of Plasmid Vectors for Direct Expression of Altered Murine Anti-CEA Heavy Chain Gene

The cysteine residues, and the resultant disulfide bonds in the region of amino acids 216-230 in the constant region of murine anti-CEA heavy chain are suspected to be important for complement fixation (Klein, et al., *Proc. Natl. Acad. Sci.*, (*USA*), 78: 524 (1981)) but not for the antigen binding property of the resulting antibody. To decrease the probability of incorrect disulfide bond formation during reconstitution according to the process of the invention herein, the nucleotides encoding the amino acid residues 226-232 which includes codons for three cysteines, are deleted as follows:

A "deleter" deoxyoligonucleotide, 5' CTAACACCATGTCAGGGT is used to delete the relevant portions of the gene from pγCEAtrp207-1☆ by the procedure of Wallace, et al., *Science,* 209: 1396 (1980) or of Adelman, et al., DNA (in press) 1983. Briefly, the "deleter" deoxyoligonucleotide is annealed with denatured pγCEAtrp207-1☆ DNA, and primer repair synthesis carried out in vitro, followed by screening by hybridization of presumptive deletion clones with ρ^{32} labelled deleter sequence.

E.5.2 Production of Cysteine Deficient Altered Antibody

The plasmid prepared in E.5.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1☆ as described above. The cells are grown, extracted for recombinant antibody chains, and the altered antibody reconstituted as described in E.1.10.

E.6 Preparation of Fab

E.6.1 Construction of a Plasmid Vector for Direct Expression of Murine Anti-CEA Gamma 1 Fab Fragment Gene pγCEAFabtrp207-1☆

FIG. **13** presents the construction of pγCEAFabtrp207-1☆. 5 μg of pBR322 was digested with Hind III, the cohesive ends made flush by treating with Klenow and dNTPs; digested with Pst I, and treated with BAP. The large vector fragment, fragment I, was recovered using 6 percent PAGE followed by electroelution.

5 μg of pγCEAtrp207-1☆ was digested with both BamH I and Pst I and the ~1570 bp DNA fragment (fragment II) containing the trp promoter and the gene sequence encoding the variable region continuing into constant region and further into the anti-CEA gamma 1 chain hinge region, was isolated and purified after electrophoresis.

Expression of the anti-CEA gamma 1 chain Fab fragment rather than complete heavy chain requires that a termination codon be constructed at the appropriate location in the gene. For this, the 260 bp Nco I-Nde I DNA fragment from 20 μg of the pγ298 was isolated and purified. A 13 nucleotide DNA primer, the complement of which encodes the last 3 C-terminal amino acids of the Fab gene and 2 bases of the 3 needed for the stop codon, was synthesized by the phosphotriester method (supra). The probe hybridizes to nucleotides 754 to 767 (FIG. **4**) which has the following sequence:

```
AspCysGlyStop
5' GGGATTGTGGTTG 3'
```

The third base of the stop codon is provided by the terminal nucleotide of the filled-in Hind III site from pBR322 cleavage described above. 500 ng of this primer was used in a primer repair reaction by phosphorylation at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl, and mixing with ~200 ng of the Nco I-Nde I DNA fragment. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, digested with BamH I and the reaction electrophoresed through a 6 percent polyacrylamide gel. ~50 ng of the 181 bp blunt end to BamH I DNA fragment, fragment III, was isolated and purified.

~100 ng of fragment I, ~100 ng each of fragments II and III were ligated overnight and transformed into *E. coli* K12 strain 294. Plasmid DNA from several tetracycline resistant transformants was analyzed for the proper construction and the nucleotide sequence through the repair blunt end filled-in Hind III junction was determined for verification of the TGA stop codon.

E.6.2 Production of Fab Protein

The plasmid prepared in E.6.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1☆ as described above. The cells are grown, extracted for recombinant antibody chains and the Fab protein reconstituted as described in E.1.10.

The invention claimed is:

1. A method for making an antibody heavy chain or fragment thereof and an antibody light chain or fragment thereof each having specificity for a desired antigen, wherein the heavy chain or fragment thereof comprises a human constant region sequence and a variable region comprising non human mammalian variable region sequences, the method comprising culturing a recombinant host cell comprising DNA encoding the heavy chain or fragment thereof and the light chain or fragment thereof and recovering the heavy chain or fragment thereof and light chain or fragment thereof from the host cell culture.

2. The method of claim **1** wherein the light chain or fragment thereof comprises a human constant region sequence and a variable region comprising non human mammalian variable region sequences.

3. The method of claim **1** wherein the host cell comprises a vector comprising DNA encoding the heavy chain or fragment thereof and DNA encoding the light chain or fragment thereof.

4. The method of claim **1** wherein the host cell comprises a vector comprising DNA encoding the heavy chain or frag-

ment thereof and a further vector comprising DNA encoding the light chain or fragment thereof.

5. The method of claim **1** wherein the non human mammalian variable region sequences are murine.

6. The method of claim **1** wherein the host cell is a prokaryotic cell.

7. The method of claim **6** wherein the prokaryotic cell is an *E. coli* cell.

8. The method of claim **1** wherein the host cell is an eukaryotic cell.

9. The method of claim **8** wherein the eukaryotic cell is a mammalian cell.

10. The method of claim **9** wherein the mammalian cell is selected from the group consisting of a VERO, HeLa, Chinese Hamster Ovary (CHO), W138, BHK, COS-7 and MDCK cell.

11. The method of claim **10** wherein the mammalian cell is a CHO cell.

12. The method of claim **10** wherein the mammalian cell is a COS-7 cell.

13. The method of claim **8** wherein the eukaryotic cell is a yeast cell.

14. The method of claim **13** wherein the yeast cell is a *Saccharomyces cerevisiae* cell.

15. A method for making an antibody or antibody fragment capable of specifically binding a desired antigen, wherein the antibody or antibody fragment comprises (a) an antibody heavy chain or fragment thereof comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences and (b) an antibody light chain or fragment thereof comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences, the method comprising coexpressing the heavy chain or fragment thereof and the light chain or fragment thereof in a recombinant host cell.

16. The method of claim **15** further comprising recovering the antibody or antibody fragment from a cell culture comprising the recombinant cell.

17. The method of claim **15** which results in the production of an antibody fragment.

18. The method of claim **17** wherein the antibody fragment is an $F(ab)_2$ fragment.

19. The method of claim **17** wherein the antibody fragment is a Fab fragment.

20. The method of claim **15** which results in the production of an antibody.

21. A method for making an antibody capable of specifically binding a desired antigen, the antibody comprising heavy and light immunoglobulin polypeptide chains each comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences, the method comprising the steps of (a) transforming a recombinant host cell with a replicable expression vector comprising DNA encoding the heavy immunoglobulin polypeptide chain and a replicable expression vector comprising DNA encoding the light immunoglobulin polypeptide chain, wherein each of the DNAs is operably linked to a promoter; and (b) culturing the host cell to produce a host cell culture that expresses said antibody.

22. A replicable expression vector comprising DNA encoding an antibody heavy chain or fragment thereof and an antibody light chain or fragment thereof each having specificity for a desired antigen, the heavy chain or fragment thereof and the light chain or fragment thereof each comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences.

23. A recombinant host cell comprising the vector of claim **22**.

24. A recombinant host cell comprising (a) a vector comprising DNA encoding an antibody heavy chain or fragment thereof comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences and (b) a vector comprising DNA encoding an antibody light chain or fragment thereof comprising a human constant region sequence and a variable region comprising non human mammalian variable region sequences.

25. A method for making an antibody heavy chain or fragment thereof and an antibody light chain or fragment thereof each having specificity for a desired antigen, wherein the heavy chain or fragment thereof comprises a variable region sequence and a human constant region sequence, the method comprising culturing a recombinant host cell comprising DNA encoding the heavy chain or fragment thereof and the light chain or fragment thereof and recovering the heavy chain or fragment thereof and light chain or fragment thereof from the host cell culture.

26. The method of claim **25** wherein the light chain or fragment thereof comprises a variable region sequence and a human constant region sequence.

27. The method of claim **25** wherein the host cell comprises a vector comprising DNA encoding the heavy chain or fragment thereof and DNA encoding the light chain or fragment thereof.

28. The method of claim **25** wherein the host cell comprises a vector comprising DNA encoding the heavy chain or fragment thereof and a further vector comprising DNA encoding the light chain or fragment thereof.

29. The method of claim **25** wherein the host cell is a prokaryotic cell.

30. The method of claim **29** wherein the prokaryotic cell is an *E. coli* cell.

31. The method of claim **25** wherein the host cell is a eukaryotic cell.

32. The method of claim **31** wherein the eukaryotic cell is a mammalian cell.

33. The method of claim **32** wherein the mammalian cell is selected from the group consisting of a VERO, HeLa, Chinese Hamster Ovary (CHO), W138, BHK, COS-7 and MDCK cell.

34. The method of claim **32** wherein the mammalian cell is a CHO cell.

35. The method of claim **32** wherein the mammalian cell is a COS-7 cell.

36. The method of claim **31** wherein the eukaryotic cell is a yeast cell.

37. The method of claim **36** wherein the yeast cell is a *Saccharomyces cerevisiae* cell.

38. A method for making an antibody or antibody fragment capable of specifically binding a desired antigen, wherein the antibody or antibody fragment comprises (a) an antibody heavy chain or fragment thereof comprising a variable region sequence and a human constant region sequence and (b) an antibody light chain or fragment thereof comprising a variable region sequence and a human constant region sequence, the method comprising coexpressing the heavy chain or fragment thereof and the light chain or fragment thereof in a recombinant host cell.

39. The method of claim **38** further comprising recovering the antibody or antibody fragment from a cell culture comprising the recombinant cell.

40. The method of claim **38** which results in the production of an antibody fragment.

41. The method of claim **38** wherein the antibody fragment is an $F(ab)_2$ fragment.

42. The method of claim **40** wherein the antibody fragment is a Fab fragment.

43. The method of claim **38** which results in the production of an antibody.

44. A method for making an antibody capable of specifically binding a desired antigen, the antibody comprising heavy and light immunoglobulin polypeptide chains each comprising a variable region sequence and a human constant region sequence, the method comprising the steps of (a) transforming a recombinant host cell with a replicable expression vector comprising DNA encoding the heavy immunoglobulin polypeptide chain and a replicable expression vector comprising DNA encoding the light immunoglobulin polypeptide chain, wherein each of the DNAs is operably linked to a promoter; and (b) culturing the host cell to produce a host cell culture that expresses said antibody.

45. A replicable expression vector comprising DNA encoding an antibody heavy chain or fragment thereof and an antibody light chain or fragment thereof each having specificity for a desired antigen, the heavy chain or fragment thereof and the light chain or fragment thereof each comprising a variable region sequence and a human constant region sequence.

46. A recombinant host cell comprising the vector of claim **45**.

47. A recombinant host cell comprising (a) a vector comprising DNA encoding an antibody heavy chain or fragment thereof comprising a variable region sequence and human constant region sequence and (b) a vector comprising DNA encoding an antibody light chain or fragment thereof comprising a variable region sequence and a human constant region sequence.

* * * * *

EXHIBIT C

US006407213B1

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Carter et al.

(10) **Patent No.: US 6,407,213 B1**
(45) **Date of Patent: Jun. 18, 2002**

(54) **METHOD FOR MAKING HUMANIZED ANTIBODIES**

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(58) **Field of Search** 435/69.6, 69.7, 435/70.21, 91, 172.2, 240.1, 240.27, 252.3, 320.1, 328; 536/23.53; 424/133.1; 530/387.3

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(57) **ABSTRACT**

Variant immunoglobulins, particularly humanized antibody polypeptides are provided, along with methods for their preparation and use. Consensus immunoglobulin sequences and structural models are also provided.

82 Claims, 9 Drawing Sheets

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FIG. 1A

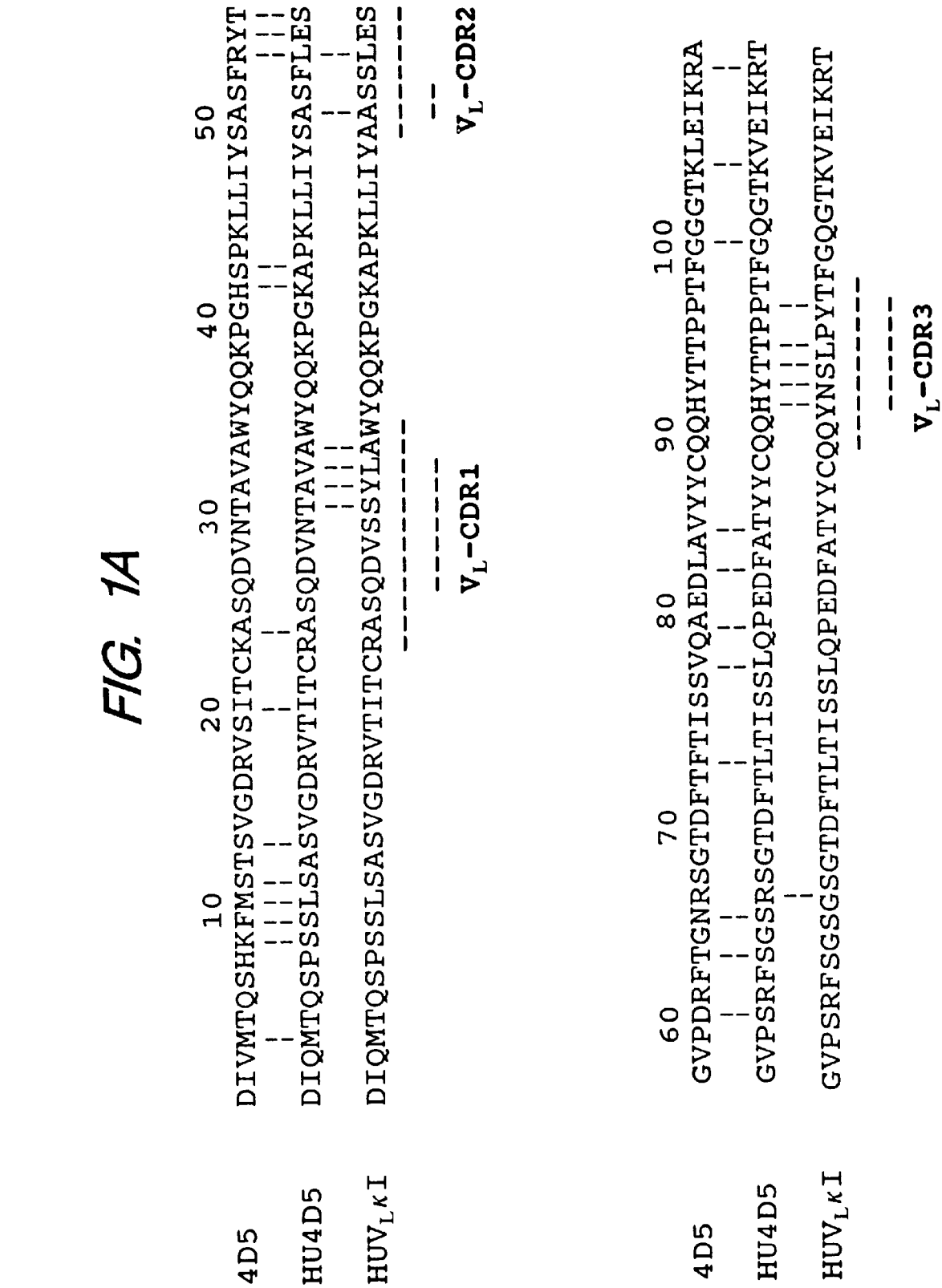
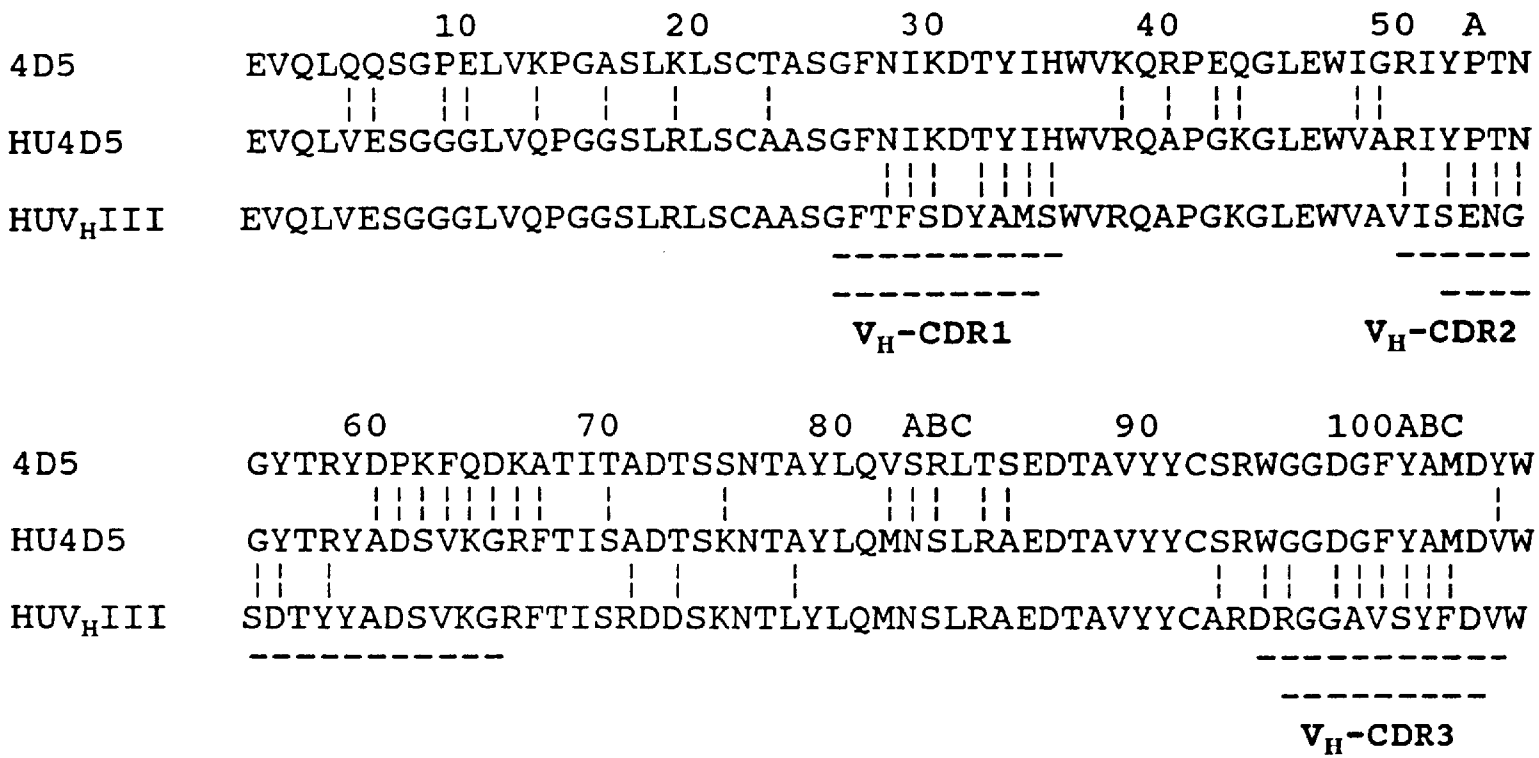

10 20 30 40 50
4D5 DIVMTQSHKFMSTSVGDRVSITCKASQDVNTAVAWYQQKPGHSPKLLIYSASFRYT
HU4D5 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLES
HUVLκI DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLES
VL-CDR1
VL-CDR2
60 70 80 90 100
4D5 GVPDRFTGNRSGTDFTFTISSVQAEDLAVYYCQQHYTTPPTFGGGTKLEIKRA
HU4D5 GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT
HUVLκI GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT
VL-CDR3

FIG. 1B



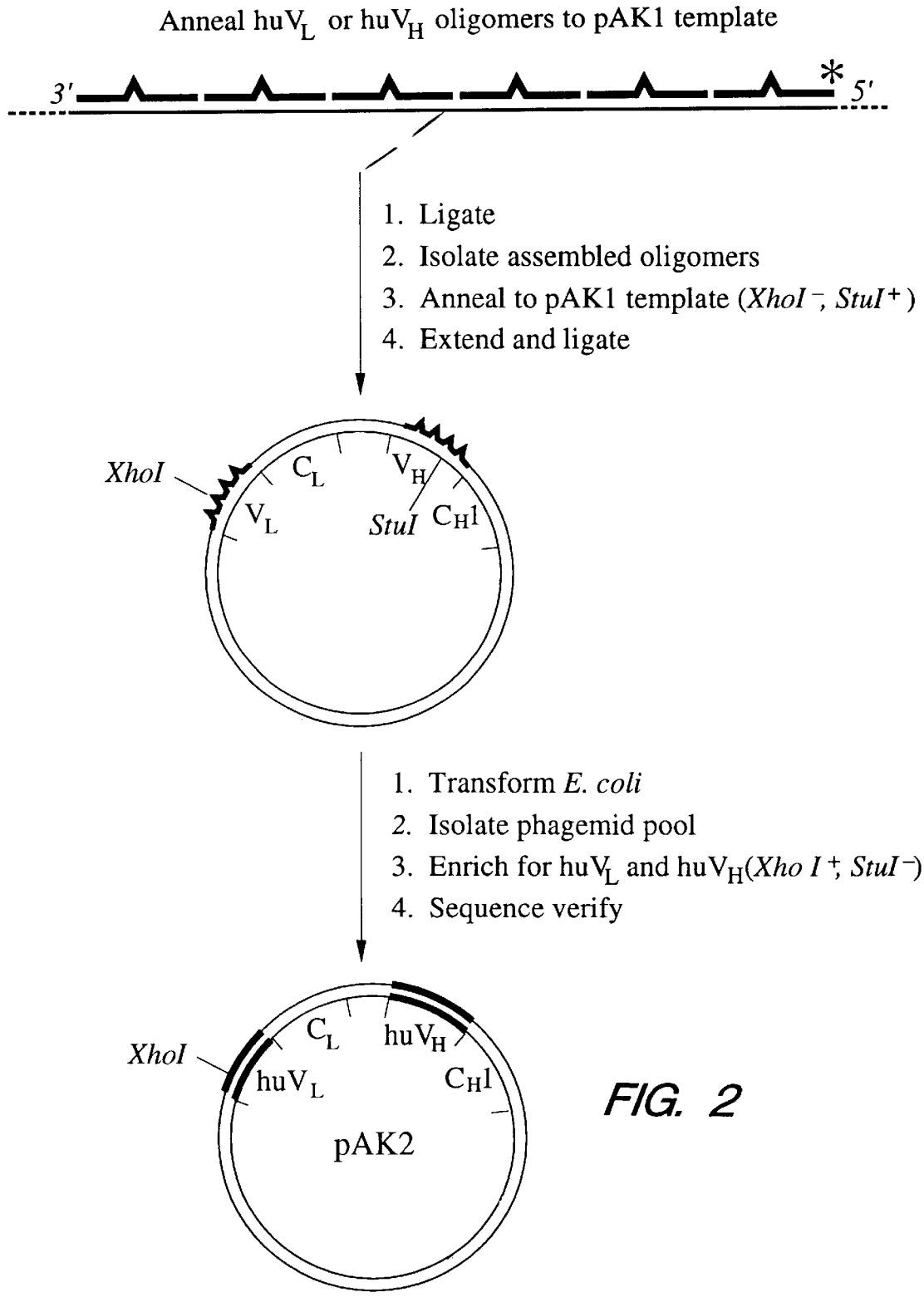

Anneal huV$_L$ or huV$_H$ oligomers to pAK1 template
3'
5'
*
1. Ligate
2. Isolate assembled oligomers
3. Anneal to pAK1 template (*XhoI*$^-$, *StuI*$^+$)
4. Extend and ligate
XhoI
C$_L$
V$_H$
V$_L$
StuI
C$_H$1
1. Transform *E. coli*
2. Isolate phagemid pool
3. Enrich for huV$_L$ and huV$_H$(*Xho I*$^+$, *StuI*$^-$)
4. Sequence verify
XhoI
C$_L$
huV$_H$
huV$_L$
C$_H$1
pAK2

FIG. 2

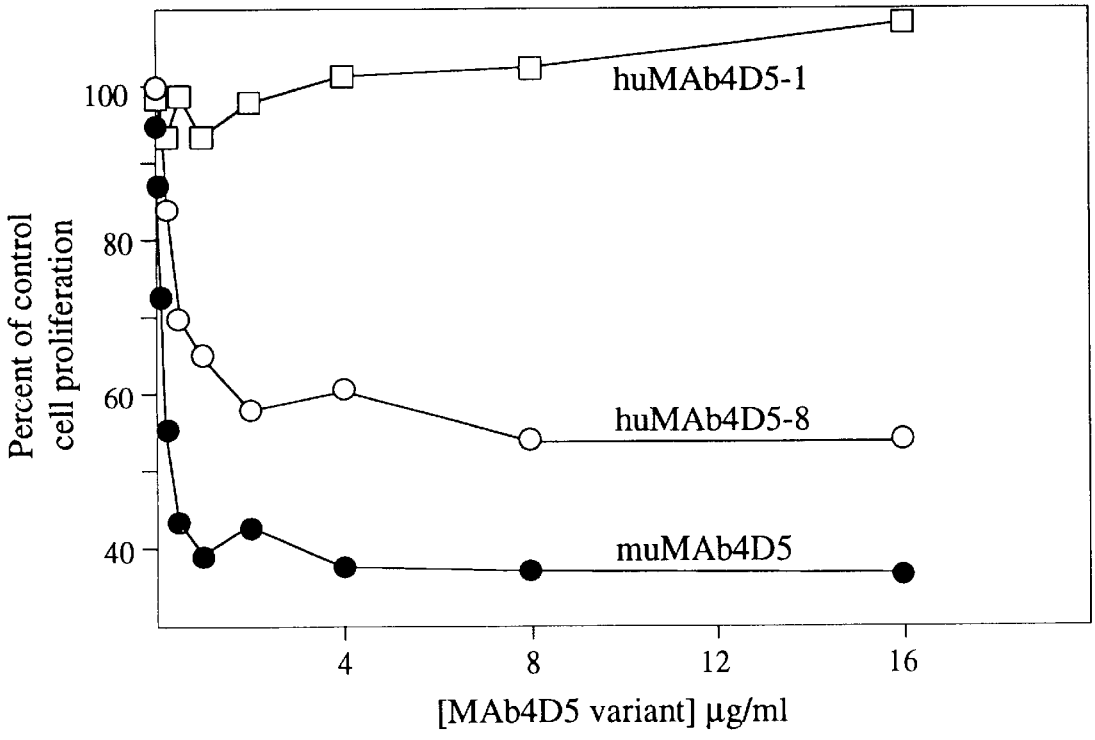

100
80
60
40
Percent of control cell proliferation
huMAb4D5-1
huMAb4D5-8
muMAb4D5
4
8
12
16
[MAb4D5 variant] µg/ml

FIG. 3

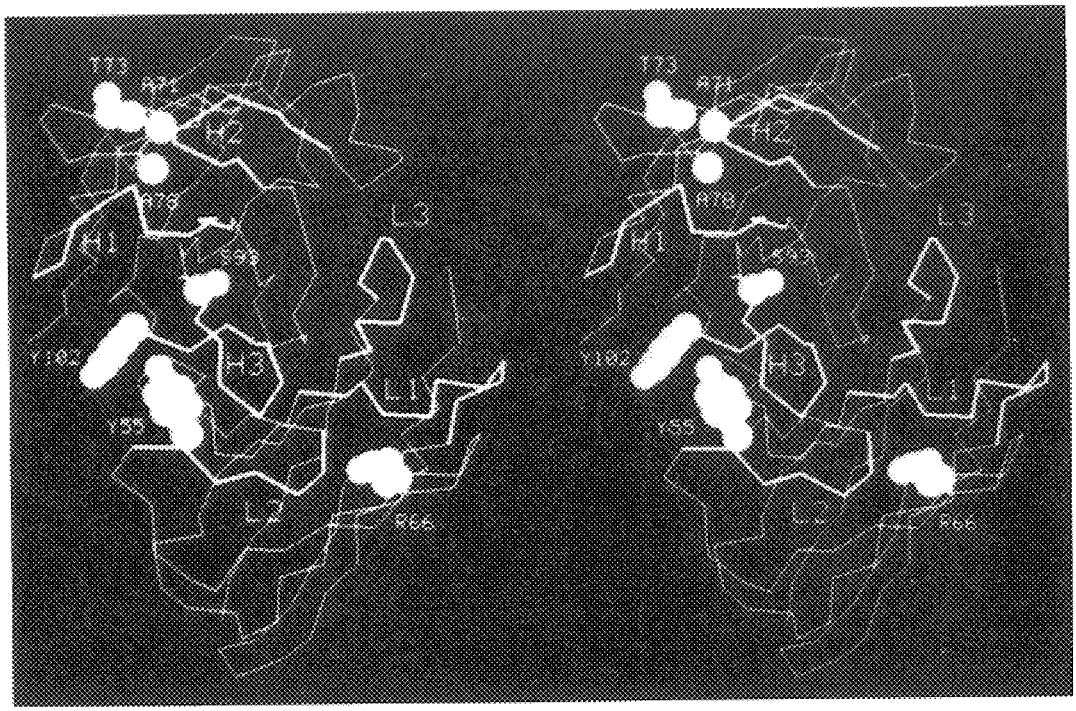


FIG. 4

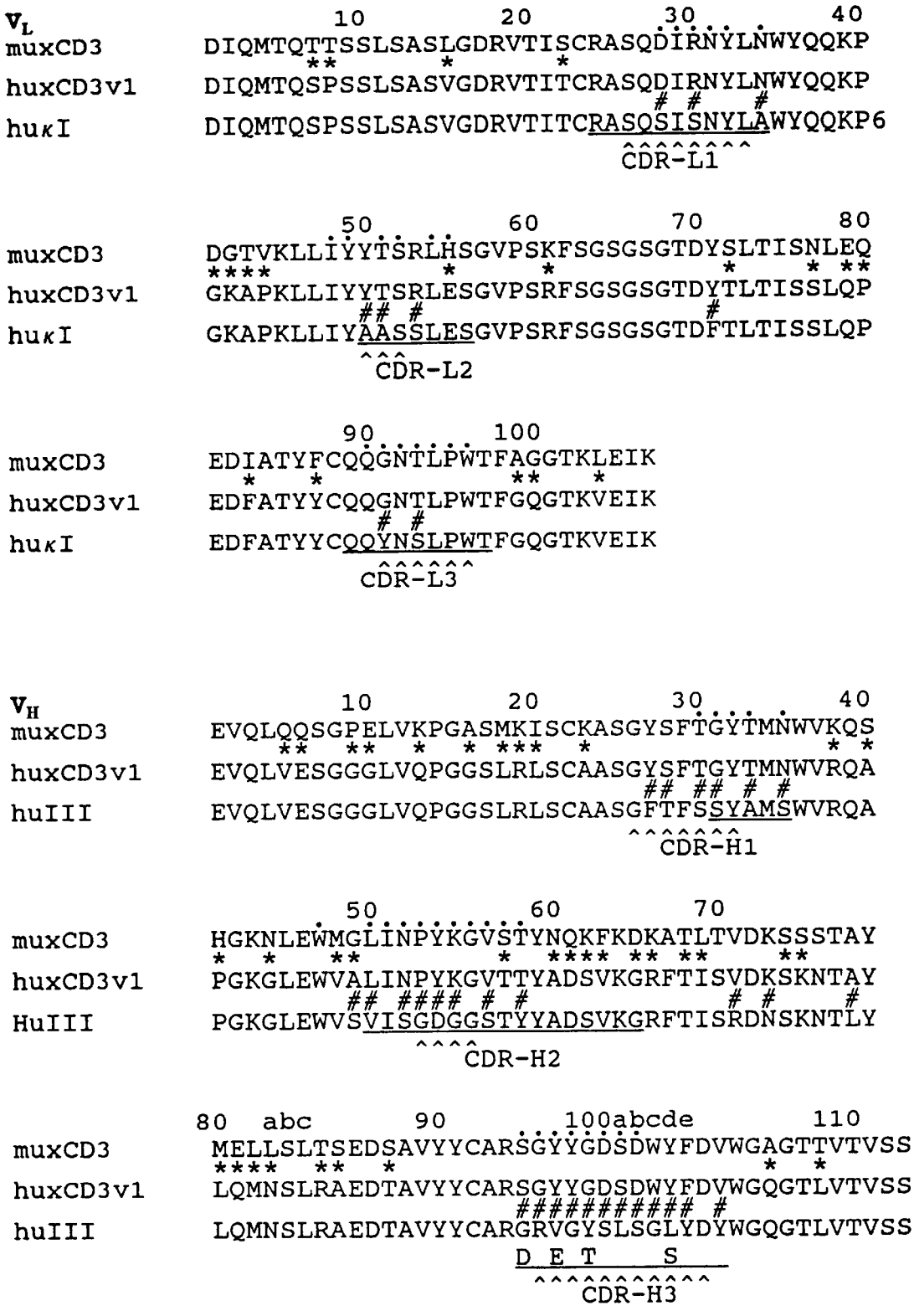

VL
muxCD3
huxCD3v1
huκI
CDR-L1
CDR-L2
CDR-L3
VH
muxCD3
huxCD3v1
huIII
CDR-H1
muxCD3
huxCD3v1
HuIII
CDR-H2
muxCD3
huxCD3v1
huIII
CDR-H3

FIG. 5

FIG. 6A-1

```
                                          10        20        30
H52H4-160                        QVQLQQSGPELVKPGASVKISCKTSGYTFTE
                                 .*** .**  **.**.*...** ********
pH52-8.0    MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCATSGYTFTE
                    10        20        30        40        50

                   40        50        60        70        80
H52H4-160   YTMHWMKQSHGKSLEWIGGFNPKNGGSSHNQRFMDKATLAVDKSTSTAYM
            ******.*. **.***..*.******.********. *..**********
pH52-8.0    YTMHWMRQAPGKGLEWVAGINPKNGGTSHNQRFMDRFTISVDKSTSTAYM
                    60        70        80        90        100

                  90       100       110       120       130
H52H4-160   ELRSLTSEDSGIYYCARWRGLNYGFDVRYFDVWGAGTTVTVSSASTKGPS
            .. ** .**...********************** ** ***********
pH52-8.0    QMNSLRAEDTAVYYCARWRGLNYGFDVRYFDVWGQGTLVTVSSASTKGPS
                   110       120       130       140       150

                 140       150       160       170       180
H52H4-160   VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
            ****** *.*** .************************************
pH52-8.0    VFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
                   160       170       180       190       200

                 190       200       210       220       230
H52H4-160   QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
            ************** **..***** ***.********** ** * *
pH52-8.0    QSSGLYSLSSVVTVTSSNFGTQTYTCNVDHKPSNTKVDKTVERKCC---V
                   210       220       230       240

                 240       250       260       270       280
H52H4-160   TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
             *******  ..**********************************.
pH52-8.0    ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQ
            250       260       270       280       290
```

FIG. 6A-2

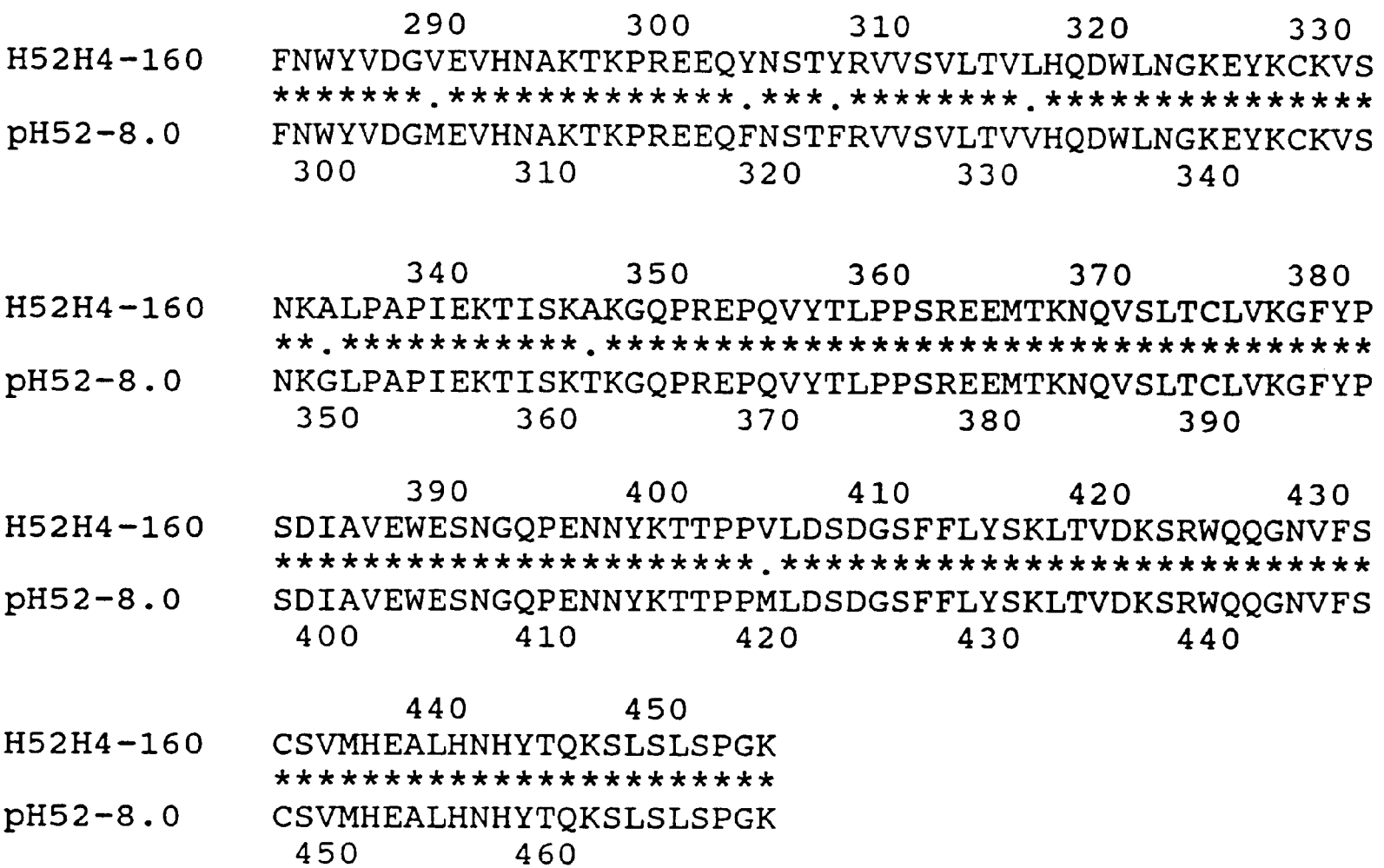


FIG. 6B

```
                                        10        20        30
H52L6-158                             DVQMTQTTSSLSASLGDRVTINCRASQDINN
                                      *.****. ******.****** *********
pH52-9.0    MGWSCIILFLVATATGVHSDIQMTQSPSSLSASVGDRVTITCRASQDINN
                    10        20        30        40        50

                   40        50        60        70        80
H52L6-158   YLNWYQQKPNGTVKLLIYYTSTLHSGVPSRFSGSGSGTDYSLTISNLDQE
            *********  . ****************************.****.*. *
pH52-9.0    YLNWYQQKPGKAPKLLIYYTSTLHSGVPSRFSGSGSGTDYTLTISSLQPE
                    60        70        80        90        100

                  90       100       110       120       130
H52L6-158   DIATYFCQQGNTLPPTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
            *.***.************ ******************************
pH52-9.0    DFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
                    110       120       130       140       150

                 140       150       160       170       180
H52L6-158   VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
            **************************************************
pH52-9.0    VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
                    160       170       180       190       200

                 190       200       210
H52L6-158    SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
             ********************************
pH52-9.0     SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
                    210       220       230
```

METHOD FOR MAKING HUMANIZED ANTIBODIES

CROSS REFERENCES

This application is a continuation-in-part of U.S. application Ser. No. 07/715,272 filed Jun. 14, 1991 (abandoned) which application is incorporated herein by reference and to which application priority is claimed under 35 USC §120.

FIELD OF THE INVENTION

This invention relates to methods for the preparation and use of variant antibodies and finds application particularly in the fields of immunology and cancer diagnosis and therapy.

BACKGROUND OF THE INVENTION

Naturally occurring antibodies (immunoglobulins) comprise two heavy chains linked together by disulfide bonds and two light chains, one light chain being linked to each of the heavy chains by disulfide bonds. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain (V_L) at one end and a constant domain at its other end, the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains, see e.g. Chothia et al., *J. Mol. Biol.* 186:651–663 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. USA* 82:4592–4596 (1985).

The constant domains are not involved directly in binding the antibody to an antigen, but are involved in various effector functions, such as participation of the antibody in antibody-dependent cellular cytotoxicity. The variable domains of each pair of light and heavy chains are involved directly in binding the antibody to the antigen. The domains of natural light and heavy chains have the same general structure, and each domain comprises four framework (FR) regions, whose sequences are somewhat conserved, connected by three hyper-variable or complementarity determining regions (CDRs) (see Kabat, E. A. et a., *Sequences of Proteins of Immunological Interest,* National Institutes of Health, Bethesda, Md., (1987)). The four framework regions largely adopt a β-sheet conformation and the CORs form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held in close proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site.

Widespread use has been made of monoclonal antibodies, particularly those derived from rodents including mice, however they are frequently antigenic in human clinical use. For example, a major limitation in the clinical use of rodent monoclonal antibodies is an anti-globulin response during therapy (Miller, R. A. et al., *Blood* 62:988–995 (1983); Schroff, R. W. et al., *Cancer Res.* 45:879–885 (1985)).

The art has attempted to overcome this problem by constructing "chimeric" antibodies in which an animal antigen-binding variable domain is coupled to a human constant domain (Cabilly et al., U.S. Pat. No. 4,816,567; Morrison, S. L. et al., *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (1984); Boulianne, G. L. et al., *Nature* 312:643–646 (1984); Neuberger, M. S. et al., *Nature* 314:268–270 (1985)). The term "chimeric" antibody is used herein to describe a polypeptide comprising at least the

antigen binding portion of an antibody molecule linked to at least part of another protein (typically an immunoglobulin constant domain).

The isotype of the human constant domain may be selected to tailor the chimeric antibody for participation in antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (see e.g. Brüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987); Riechmann, L. et al., *Nature* 332:323–327 (1988); Love et al., *Methods in Enzymology* 178:515–527 (1989); Bindon et al., *J. Exp. Med.* 168:127–142 (1988).

In the typical embodiment, such chimeric antibodies contain about one third rodent (or other non-human species) sequence and thus are capable of eliciting a significant anti-globulin response in humans. For example, in the case of the murine anti-CD3 antibody, OKT3, much of the resulting anti-globulin response is directed against the variable region rather than the constant region (Jaffers, G. J. et a., *Transplantation* 41:572–578 (1986)).

In a further effort to resolve the antigen binding functions of antibodies and to minimize the use of heterologous sequences in human antibodies, Winter and colleagues (Jones, P. T. et al., *Nature* 321:522–525 (1986); Riechmann, L. et al., *Nature* 332:323–327 (1988); Verhoeyen, M. et al., *Science* 239:1534–1536 (1988)) have substituted rodent CDRs or CDR sequences for the corresponding segments of a human antibody. As used herein, the term "humanized" antibody is an embodiment of chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The therapeutic promise of this approach is supported by the clinical efficacy of a humanized antibody specific for the CAMPATH-1 antigen with two non-Hodgkin lymphoma patients, one of whom had previously developed an anti-globulin response to the parental rat antibody (Riechmann, L. et al., *Nature* 332:323–327 (1988); Hale, G. et al., *Lancet i:*1394–1399 (1988)). A murine antibody to the interleukin 2 receptor has also recently been humanized (Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)) as a potential immunosuppressive reagent. Additional references related to humanization of antibodies include Co et al., *Proc. Natl. Acad. Sci. USA* 88:2869–2873 (1991); Gorman et al., *Proc. Natl. Acad. Sci. USA* 88:4181–4185 (1991); Daugherty et al., *Nucleic Acids Research* 19(9):2471–2476 (1991); Brown et al., *Proc. Natl. Acad. Sci. USA* 88:2663–2667 (1991); Junghans et al., *Cancer Research* 50:1495–1502 (1990).

In some cases, substituting CDRs from rodent antibodies for the human CDRs in human frameworks is sufficient to transfer high antigen binding affinity (Jones, P. T. et al., *Nature* 321:522–525 (1986); Verhoeyen, M. et al., *Science* 239:1534–1536 (1988)), whereas in other cases it has been necessary to additionally replace one (Riechmann, L. et al., *Nature* 332:323–327 (1988)) or several (Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)) framework region (FR) residues. See also Co et al., supra.

For a given antibody a small number of FR residues are anticipated to be important for antigen binding. Firstly for example, certain antibodies have been shown to contain a few FR residues which directly contact antigen in crystal structures of antibody-antigen complexes (e.g., reviewed in Davies, D. R. et al., *Ann. Rev. Biochem.* 59:439–473 (1990)).

Secondly, a number of FR residues have been proposed by Chothia, Lesk and colleagues (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987); Chothia, C. et al., *Nature* 342:877–883 (1989); Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) as critically affecting the conformation of particular CDRs and thus their contribution to antigen binding. See also Margolies et al., *Proc. Natl. Acad. Sci. USA* 72:2180–2184 (1975).

It is also known that, in a few instances, an antibody variable domain (either V_H or V_L) may contain glycosylation sites, and that this glycosylation may improve or abolish antigen binding, Pluckthun, *Biotechnology* 9:545–51 (1991); Spiegelberg et al., *Biochemistry* 9:4217–4223 (1970); Wallic et al., *J. Exp. Med.* 168:1099–1109 (1988); Sox et al., *Proc. Natl. Acad. Sci. USA* 66:975–982 (1970); Margni et al., *Ann. Rev. Immunol* 6:535–554 (1988). Ordinarily, however, glycosylation has no influence on the antigen-binding properties of an antibody, Pluckthun, supra, (1991).

The three-dimensional structure of immunoglobulin chains has been studied, and crystal structures for intact immunoglobulins, for a variety of immunoglobulin fragments, and for antibody-antigen complexes have been published (see e.g., Saul et al., *Journal of Biological Chemistry* 25:585–97 (1978); Sheriff et al., *Proc. Natl. Acad. Sci. USA* 84:8075–79 (1987); Segal et al., *Proc. Natl. Acad. Sci. USA* 71:4298–4302 (1974); Epp et al., *Biochemistry* 14(22) :4943–4952 (1975); Marquart et al., *J. Mol. Biol.* 141:369–391 (1980); Furey et al., *J. Mol. Biol.* 167:661–692 (1983); Snow and Amzel, *Protein: Structure, Function, and Genetics* 1:267–279, Alan R. Liss, Inc. pubs. (1986); Chothia and Lesk, *J. Mol. Bol.* 196:901–917 (1987); Chothia et al., *Nature* 342:877–883 (1989); Chothia et al., *Science* 233:755–58 (1986); Huber et al., *Nature* 264:415–420 (1976); Bruccoleri et al., *Nature* 335:564–568 (1988) and *Nature* 336:266 (1988); Sherman et al., *Journal of Biological Chemistry* 263:4064–4074 (1988); Amzel and Poljak, *Ann. Rev. Biochem.* 48:961–67 (1979); Silverton et al., *Proc. Natl. Acad. Sci. USA* 74:5140–5144 (1977); and Gregory et al., *Molecular Immunology* 24:821–829 (1987). It is known that the function of an antibody is dependent on its three dimensional structure, and that amino acid substitutions can change the three-dimensional structure of an antibody, Snow and Amzel, supra. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., *Nature* 332:323–327 (1988); Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)).

Humanizing an antibody with retention of high affinity for antigen and other desired biological activities is at present difficult to achieve using currently available procedures. Methods are needed for rationalizing the selection of sites for substitution in preparing such antibodies and thereby increasing the efficiency of antibody humanization.

The proto-oncogene HER2 (human epidermal growth factor receptor 2) encodes a protein tyrosine kinase ($p185^{HER2}$) that is related to and somewhat homologous to the human epidermal growth factor receptor (see Coussens, L. et al., *Science* 230:1132–1139 (1985); Yamamoto, T. et al., *Nature* 319:230–234 (1986); King, C. R. et al., *Science* 229:974–976 (1985)). HER2 is also known in the field as c-erbB-2, and sometimes by the name of the rat homolog, neu. Amplification and/or overexpression of HER2 is associated with multiple human malignancies and appears to be integrally involved in progression of 25–30% of human breast and ovarian cancers (Slamon, D. J. et al., *Science* 235:177–182 (1987), Slamon, D. J. et al., *Science* 244:707–712 (1989)). Furthermore, the extent of amplification is inversely correlated with the observed median patient survival time (Slamon, supra, Science 1989).

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)), directed against the extracellular domain (ECD) of $p185^{HER2}$, specifically inhibits the growth of tumor cell lines overexpressing $p185^{HER2}$ in monolayer culture or in soft agar (Hudziak, R. M. et al., *Molec. Cell. Biol* 9:1165–1172 (1989); Lupu, R. et al., *Science* 249:1552–1555 (1990)). MuMAb4D5 also has the potential of enhancing tumor cell sensitivity to tumor necrosis factor, an important effector molecule in macrophage-mediated tumor cell cytotoxicity (Hudziak, supra, 1989; Shepard, H. M. and Lewis, G. D. *J. Clinical Immunology* 8:333–395 (1988)). Thus muMAb4D5 has potential for clinical intervention in and imaging of carcinomas in which $p185^{HER2}$ is overexpressed. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. However, this antibody may be immunogenic in humans.

It is therefore an object of this invention to provide methods for the preparation of antibodies which are less antigenic in humans than non-human antibodies but have desired antigen binding and other characteristics and activities.

It is a further object of this invention to provide methods for the efficient humanization is of antibodies, i.e. selecting non-human amino acid residues for importation into a human antibody background sequence in such a fashion as to retain or improve the affinity of the non-human donor antibody for a given antigen.

It is another object of this invention to provide humanized antibodies capable of binding $p185^{HER2}$.

Other objects, features, and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

SUMMARY OF THE INVENTION

The objects of this invention are accomplished by a method for making a humanized antibody comprising amino acid sequence of an import, non-human antibody and a human antibody, comprising the steps of:

a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus variable domain;

b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

1. non-covalently binds antigen directly,

2. interacts with a CDR; or

3. participates in the V_L–V_H interface; and

g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, the method of this invention comprises the additional steps of determining if any non-homologous residues identified in step (e) are exposed on the surface of the domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), retaining the consensus residue.

Additionally, in certain embodiments the method of this invention comprises the feature wherein the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71 L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)).

In certain embodiments, the method of this invention comprises the additional steps of searching either or both of the import, non-human and the consensus variable domain sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid residue that binds to antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antibody affinity). If the import sequence bears the glycosylation site, it is preferred to substitute that site for the corresponding residues in the consensus human if the glycosylation site is reasonably expected to be important. If only the consensus sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

Another embodiment of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

Certain alternate embodiments of the methods of this invention comprise obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:

a. (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, or

b. (in the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 78H, 91H, 92H, 93H, and 103H.

In preferred embodiments, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody.

Optionally, this just-recited embodiment comprises the additional steps of following the method steps appearing at the beginning of this summary and determining whether a particular amino acid residue can reasonably be expected to have undesirable effects.

This invention also relates to a humanized antibody comprising the CDR sequence of an import, non-human antibody and the FR sequence of a human antibody, wherein an amino acid residue within the human FR sequence located at any one of the sites 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H has been substituted by another residue. In preferred embodiments, the residue substituted at the human FR site is the residue found at the corresponding location of the non-human antibody from which the non-human CDR was obtained. In other embodiments, no human FR residue other than those set forth in this group has been substituted.

This invention also encompasses specific humanized antibody variable domains, and isolated polypeptides having homology with the following sequences.

1. SEQ. ID NO. 1, which is the light chain variable domain of a humanized version of muMAb4D5: DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLESGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT

2. SEQ. ID NO. 2, which is the heavy chain variable domain of a humanized version of muMAb4D5): EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLVTVSS

In another aspect, this invention provides a consensus antibody variable domain amino acid sequence for use in the preparation of humanized antibodies, methods for obtaining, using, and storing a computer representation of such a consensus sequence, and computers comprising the sequence data of such a sequence. In one embodiment, the following consensus antibody variable domain amino acid sequences are provided:

SEQ. ID NO. 3 (light chain): DDIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT, and

SEQ. ID NO. 4 (heavy chain): EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAWYCSRWGGDGFYAMDVWGQGTLVTVSS

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**A shows the comparison of the V_L domain amino acid residues of muMAb4D5, huMAb4D5, and a consensus sequence (FIG. **1**A, SEQ.ID NO. 5, SEQ. ID NO. 1 and SEQ. ID NO. 3, respectively). FIG. **1**B shows the comparison between the V_H domain amino acid residues of the muMAb4D5, huMAb4D5, and a consensus sequence (FIG. **1**B, SEQ. ID NO. 6, SEQ. ID NO. 2 and SEQ. ID NO. 4, respectively). Both FIGS. **1**A and **1**B use the generally accepted numbering scheme from Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987)). In both FIG. **1**A

and FIG. **1**B, the CDR residues determined according to a standard sequence definition (as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) are indicated by the first underlining beneath the sequences, and the CDR residues determined according to a structural definition (as in Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)) are indicated by the second, lower underlines. The mismatches between genes are shown by the vertical lines.

FIG. **2** shows a scheme for humanization of muMAb4D5 V_L and V_H by gene conversion mutagenesis.

FIG. **3** shows the inhibition of SK-BR-3 proliferation by MAb4D5 variants. Relative cell proliferation was determined as described (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165–1172 (1989)) and data (average of triplicate determinations) are presented as a percentage of results with untreated cultures for muMAb4D5 (●), huMAb4D5-8 (○) and huMAb4D5-1 (□).

FIG. **4** shows a stereo view of α-carbon tracing for a model of huMAb4D5-8 V_L and V_H. The CDR residues (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) are shown in bold and side chains of V_H residues A71, T73, A78, S93, Y102 and V_L residues Y55 plus R66 (see Table 3) are shown.

FIG. **5** shows an amino acid sequence comparison of V_L (top panel) and V_H (lower panel) domains of the murine anti-CD3 monoclonal Ab UCHT1 (muxCD3, Shalaby et al., *J. Exp. Med.* 175, 217–225 (1992) with a humanized variant of this antibody (huxCD3v1). Also shown are consensus sequences (most commonly occurring residue or pair of residues) of the most abundant human subgroups, namely V_L κ 1 and V_H III upon which the humanized sequences are based (Kabat, E. A. et al., *Sequences of Proteins of immunological Interest,* 5th edition, National Institutes of Health, Bethesda, Md., USA (1991)). The light chain sequences—muxCD3, huxCD3v1 and huKI—correspond to SEQ.ID. NOs 16, 17, and 18, respectively. The heavy chain sequences—muxCD3, huxCD3v1 and huxI—correspond to SEQ.ID.NOs 19, 26, and 21, respectively. Residues which differ between muxCD3 and huxCD3v1 are identified by an asterisk (*), whereas those which differ between humanized and consensus sequences are identified by a sharp sign (#). A bullet (•) denotes that a residue at this position has been found to contact antigen in one or more crystallographic structures of antibody/antigen complexes (Kabat et al., 1991; Mian, I. S. et al., *J. Mol. Biol* 217, 133–151 (1991)). The location of CDR residues according to a sequence definition (Kabat et al., 1991) and a structural definition (Chothia and Lesk, supra 1987) are shown by a line and carats (^) beneath the sequences, respectively.

FIG. **6**A compares murine and humanized amino acid sequences for the heavy chain of an anti-CD18 antibody. H52H4-160 (SEQ. ID. NO. 22) is the murine sequence, and pH52–8.0 (SEQ. ID. NO. 23) is the humanized heavy chain sequence. pH52–8.0 residue 143S is the final amino acid in the variable heavy chain domain V_H, and residue 144A is the first amino acid in the constant heavy chain domain C_{H1}.

FIG. **6**B compares murine and humanized amino acid sequences for the light chain of an anti-CD18 antibody. H52L6-158 (SEQ. ID. NO. 24) is the murine sequence, and pH52–9.0 (SEQ. ID. NO. 25) is the humanized light chain sequence. pH52–9.0 residue 128T is the final amino acid in the light chain variable domain V_L, and residue 129V is the first amino acid in the light chain constant domain C_L.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In general, the following words or phrases have the indicated definitions when used in the description, examples, and claims:

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) is directed against the extracellular domain (ECD) of $p185^{HER2}$. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. In this description and claims, the terms muMAb4D5, chMAb4D5 and huMAb4D5 represent murine, chimerized and humanized versions of the monoclonal antibody 4D5, respectively.

A humanized antibody for the purposes herein is an immunoglobulin amino acid sequence variant or fragment thereof which is capable of binding to a predetermined antigen and which comprises a FR region having substantially the amino acid sequence of a human immunoglobulin and a CDR having substantially the amino acid sequence of a non-human immunoglobulin.

Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are referred to herein as "import" residues, which are typically taken from an "import" antibody domain, particularly a variable domain. An import residue, sequence, or antibody has a desired affinity and/or specificity, or other desirable antibody biological activity as discussed herein.

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, Fabc, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Ordinarily, the antibody will contain both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain.

The humanized antibody will be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and lgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG_1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG_2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art.

The FR and CDR regions of the humanized antibody need not correspond precisely to the parental sequences, e.g., the import CDR or the consensus FR may be mutagenized by substitution, insertion or deletion of at least one residue so that the CDR or FR residue at that site does not correspond to either the consensus or the import antibody. Such mutations, however, will not be extensive. Usually, at least 75% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences, more often 90%, and most preferably greater than 95%.

In general, humanized antibodies prepared by the method of this invention are produced by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those

skilled in the art. Computer programs are available which illustrate and display probable three dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen.

Residues that influence antigen binding are defined to be residues that are substantially responsible for the antigen affinity or antigen specificity of a candidate immunoglobulin, in a positive or a negative sense. The invention is directed to the selection and combination of FR residues from the consensus and import sequence so that the desired immunoglobulin characteristic is achieved. Such desired characteristics include increases in affinity and greater specificity for the target antigen, although it is conceivable that in some circumstances the opposite effects might be desired. In general, the CDR residues are directly and most substantially involved in influencing antigen binding (although not all CDR residues are so involved and therefore need not be substituted into the consensus sequence). However, FR residues also have a significant effect and can exert their influence in at least three ways: They may noncovalently directly bind to antigen, they may interact with CDR residues and they may affect the interface between the heavy and light chains.

A residue that noncovalently directly binds to antigen is one that, by three dimensional analysis, is reasonably expected to noncovalently directly bind to antigen. Typically, it is necessary to impute the position of antigen from the spatial location of neighboring CDRs and the dimensions and structure of the target antigen. In general, only those humanized antibody residues that are capable of forming salt bridges, hydrogen bonds, or hydrophobic interactions are likely to be involved in non-covalent antigen binding, however residues which have atoms which are separated from antigen spatially by 3.2 Angstroms or less may also non-covalently interact with antigen. Such residues typically are the relatively larger amino acids having the side chains with the greatest bulk, such as tyrosine, arginine, and lysine. Antigen-binding FR residues also typically will have side chains that are oriented into an envelope surrounding the solvent oriented face of a CDR which extends about 7 Angstroms into the solvent from the CDR domain and about 7 Angstroms on either side of the CDR domain, again as visualized by three dimensional modeling.

A residue that interacts with a CDR generally is a residue that either affects the conformation of the CDR polypeptide backbone or forms a noncovalent bond with a CDR residue side chain. Conformation-affecting residues ordinarily are those that change the spatial position of any CDR backbone atom (N, Ca, C, O, Cβ) by more than about 0.2 Angstroms. Backbone atoms of CDR sequences are displaced for example by residues that interrupt or modify organized structures such as beta sheets, helices or loops. Residues that can exert a profound affect on the conformation of neighboring sequences include proline and glycine, both of which are capable of introducing bends into the backbone. Other residues that can displace backbone atoms are those that are capable of participating in salt bridges and hydrogen bonds.

A residue that interacts with a CDR side chain is one that is reasonably expected to form a noncovalent bond with a CDR side chain, generally either a salt bridge or hydrogen bond. Such residues are identified by three dimensional positioning of their side chains. A salt or ion bridge could be expected to form between two side chains positioned within about 2.5–3.2 Angstroms of one another that bear opposite charges, for example a lysinyl and a glutamyl pairing. A hydrogen bond could be expected to form between the side chains of residue pairs such as seryl or threonyl with aspartyl or glutamyl (or other hydrogen accepting residues). Such pairings are well known in the protein chemistry art and will be apparent to the artisan upon three dimensional modeling of the candidate immunoglobulin.

Immunoglobulin residues that affect the interface between heavy and light chain variable regions ("the V_L–V_H interface") are those that affect the proximity or orientation of the two chains with respect to one another. Certain residues involved in interchain interactions are already known and include V_L residues 34, 36, 38, 44, 46, 87, 89, 91, 96, and 98 and V_H residues 35, 37, 39, 45, 47, 91, 93, 95, 100, and 103 (utilizing the nomenclature setforth in Kabat et al., *Sequences of Proteins of immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)). Additional residues are newly identified by the inventors herein, and include 43L, 85L, 43H and 60H. While these residues are indicated for IgG only, they are applicable across species. In the practice of this invention, import antibody residues that are reasonably expected to be involved in interchain interactions are selected for substitution into the consensus sequence. It is believed that heretofore no humanized antibody has been prepared with an intrachain-affecting residue selected from an import antibody sequence.

Since it is not entirely possible to predict in advance what the exact impact of a given substitution will be it may be necessary to make the substitution and assay the candidate antibody for the desired characteristic. These steps, however, are per se routine and well within the ordinary skill of the art.

CDR and FR residues are determined according to a standard sequence definition (Kabat et al., *Sequences of Proteins of Immunological Interest,* National Institutes of Health, Bethesda Md. (1987), and a structural definition (as in Chothia and Lesk, *J. Mol. Biol.* 196:901–917 (1987). Where these two methods result in slightly different identifications of a CDR, the structural definition is preferred, but the residues identified by the sequence definition method are considered important FR residues for determination of which framework residues to import into a consensus sequence.

Throughout this description, reference is made to the numbering scheme from Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987) and (1991). In these compendiums, Kabat lists many amino acid sequences for antibodies for each subclass, and lists the most commonly occurring amino acid for each residue position in that subclass. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. The Kabat numbering scheme is followed in this description.

For purposes of this invention, to assign residue numbers to a candidate antibody amino acid sequence which is not included in the Kabat compendium, one follows the following steps. Generally, the candidate sequence is aligned with any immunoglobulin sequence or any consensus sequence in Kabat. Alignment may be done by hand, or by computer using commonly accepted computer programs; an example of such a program is the Align 2 program discussed in this description. Alignment may be facilitated by using some amino acid residues which are common to most Fab

sequences. For example, the light and heavy chains each typically have two cysteines which have the same residue numbers; in V_L domain the two cysteines are typically at residue numbers 23 and 88, and in the V_H domain the two cysteine residues are typically numbered 22 and 92. Framework residues generally, but not always, have approximately the same number of residues, however the CDRs will vary in size. For example, in the case of a CDR from a candidate sequence which is longer than the CDR in the sequence in Kabat to which it is aligned, typically suffixes are added to the residue number to indicate the insertion of additional residues (see, e.g. residues 100abcde in FIG. **5**). For candidate sequences which, for example, align with a Kabat sequence for residues 34 and 36 but have no residue between them to align with residue 35, the number 35 is simply not assigned to a residue.

Thus, in humanization of an import variable sequence, where one cuts out an entire human or consensus CDR and replaces it with an import CDR sequence, (a) the exact number of residues may be swapped, leaving the numbering the same, (b) fewer import amino acid residues may be introduced than are cut, in which case there will be a gap in the residue numbers, or (c) a larger number of amino acid residues may be introduced then were cut, in which case the numbering will involve the use of suffixes such as 100abcde.

The terms "consensus sequence" and "consensus antibody" as used herein refers to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all immunoglobulins of any particular subclass or subunit structure. The consensus sequence may be based on immunoglobulins of a particular species or of many species. A "consensus" sequence, structure, or antibody is understood to encompass a consensus human sequence as described in certain embodiments of this invention, and to refer to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all human immunoglobulins of any particular subclass or subunit structure. This invention provides consensus human structures and consensus structures which consider other species in addition to human.

The subunit structures of the live immunoglobulin classes in humans are as follows:

Class	Heavy Chain	Subclasses	Light Chain	Molecular Formula
IgG	γ	γ1, γ2, γ3, γ4	κ or λ	$(\gamma_2\kappa_2)$, $(\gamma_2\lambda_2)$
IgA	α	α1, α2	κ or λ	$(\alpha_2\kappa_2)_n$[8], $(\alpha_2\lambda_2)_n$[8]
IgM	μ	none	κ or λ	$(\mu_2\kappa_2)_5$, $(\mu_2\lambda_2)_5$
IgD	δ	none	κ or λ	$(\delta_2\kappa_2)$, $(\delta_2\lambda_2)$
IgE	ϵ	none	κ or λ	$(\epsilon_2\kappa_2)$, $(\epsilon_2\lambda_2)$

([8]n may equal 1, 2, or 3)

In preferred embodiments of an IgGγl human consensus sequence, the consensus variable domain sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al., *Sequences of Proteins of Immunological Interest,* National Institutes of Health, Bethesda Md. (1987), namely V_L κ subgroup I and V_H group III. In such preferred embodiments, the V_L consensus domain has the amino acid sequence:

DIQMTQSPSSLSASVGDRVTITCRASQD-VSSYLAWYQQKPGKAPKLLIYAASSLES-GVPSRFSGSGSGTDFTLTISSLQPEDFA-TYYCQQYNSLPYTFGQGTKVEIKRT (SEQ. ID NO. 3);

the V_H consensus domain has the amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSW VRQAPGKGLEWVAVISENGGYTRYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD GFYAMDVWGQGTLVTVSS (SEQ. ID NO. 4).

These sequences include consensus CDRs as well as consensus FR residues (see for example in FIG. **1**).

While not wishing to be limited to any particular theories, it may be that these preferred embodiments are less likely to be immunogenic in an individual than less abundant subclasses. However, in other embodiments, the consensus sequence is derived from other subclasses of human immunoglobulin variable domains. In yet other embodiments, the consensus sequence is derived from human constant domains.

Identity or homology with respect to a specified amino acid sequence of this invention is defined herein as the percentage of amino acid residues in a candidate sequence that are identical with the specified residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into the specified sequence shall be construed as affecting homology. All sequence alignments called for in this invention are such maximal homology alignments. While such alignments may be done by hand using conventional methods, a suitable computer program is the "Align 2" program for which protection is being sought from the U.S. Register of Copyrights (Align 2, by Genentech, Inc., application filed Dec. 9, 1991).

"Non-homologous" import antibody residues are those residues which are not identical to the amino acid residue at the analogous or corresponding location in a consensus sequence, after the import and consensus sequences are aligned.

The term "computer representation" refers to information which is in a form that can be manipulated by a computer. The act of storing a computer representation refers to the act of placing the information in a form suitable for manipulation by a computer.

This invention is also directed to novel polypeptides, and in certain aspects, isolated novel humanized anti-$p185^{HER2}$ antibodies are provided. These novel anti-$p185^{HER2}$ antibodies are sometimes collectively referred to herein as huMAb4D5, and also sometimes as the light or heavy chain variable domains of huMAb4D5, and are defined herein to be any polypeptide sequence which possesses a biological property of a polypeptide comprising the following polypeptide sequence:

DIQMTQSPSSLSASVGDRVTITCRASODVNTAVAWY QQKPGKAPKLLIYSASFLESGVPSRFSGSRSGT DFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK VEIKRT (SEQ. ID NO. 1, which is the light chain variable domain of huMAb4D5); or

EVQLVESGGGLVOPGGSLRLSCAASGFNIKDTYIHW VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD GFYAMDVWGQGTLVTVSS (SEQ. ID NO. 2, which is the heavy chain variable domain of huMAb4D5).

"Biological property", as relates for example to anti-$p185^{HER2}$, for the purposes herein means an in vivo effector or antigen-binding function or activity that is directly or indirectly performed by huMAb4D5 (whether in its native or denatured conformation). Effector functions include $p185^{HER2}$ binding, any hormonal or hormonal antagonist activity, any mitogenic or agonist or antagonist activity, any

cytotoxic activity. An antigenic function means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the polypeptide sequence of huMAb4D5.

Biologically active huMAb4D5 is defined herein as a polypeptide that shares an effector function of huMAb4D5. A principal known effector function of huMAb4D5 is its ability to bind to $p185^{HER2}$.

Thus, the biologically active and antigenically active huMAb4D5 polypeptides that are the subject of certain embodiments of this invention include the sequence of the entire translated nucleotide sequence of huMAb4D5; mature huMAb4D5; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues comprising sequences from muMAb4D5 plus residues from the human FR of huMAb4D5; amino acid sequence variants of huMAb4D5 wherein an amino acid residue has been inserted N- or C-terminal to, or within, huMAb4D5 or its fragment as defined above; amino acid sequence variants of huMAb4D5 or its fragment as defined above wherein an amino acid residue of huMAb4D5 or its fragment as defined above has been substituted by another residue, including predetermined mutations by, e.g., site-directed or PCR mutagenesis; derivatives of huMAb4D5 or its fragments as defined above wherein huMAb4D5 or its fragments have been covalent modified, by substitution, chemical, enzymatic, or other appropriate means, with a moiety other than a naturally occurring amino acid; and glycosylation variants of huMAb4D5 (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of suitable residues). Such fragments and variants exclude any polypeptide heretofore identified, including muMAb4D5 or any known polypeptide fragment, which are anticipatory order 35 U.S.C. 102 as well as polypeptides obvious thereover under 35 U.S.C. 103.

An "isolated" polypeptide means polypeptide which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, for example, a polypeptide product comprising huMAb4D5 will be purified from a cell culture or other synthetic environment (1) to greater than 95% by weight of protein as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a gas- or liquid-phase sequenator (such as a commercially available Applied Biosystems sequenator Model 470, 477, or 473), or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated huMAb4D5 includes huMAb4D5 in situ within recombinant cells since at least one component of the huMAb4D5 natural environment will not be present. Ordinarily, however, isolated huMAb4D5 will be prepared by at least one purification step.

In accordance with this invention, huMAb4D5 nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active huMAb4D5, is complementary to nucleic acid sequence encoding such huMAb4D5, or hybridizes to nucleic acid sequence encoding such huMAb4D5 and remains stably bound to it under stringent conditions, and comprises nucleic acid from a muMAb4D5 CDR and a human FR region.

Preferably, the huMAb4D5 nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably at 90%, and most preferably 95%, with the huMAb4D5 amino acid sequence. Preferably, a nucleic acid molecule that hybridizes to the huMAb4D5 nucleic acid contains at least 20, more preferably 40, and most preferably 90 bases. Such hybridizing or complementary nucleic acid, however, is further defined as being novel under 35 U.S.C. 102 and unobvious under 35 U.S.C. 103 over any prior art nucleic acid.

Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0/1% $NaDodSO_4$ at 50° C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serumalbumin/0/1% Ficoll/0/1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C.; or (3) employ 50% formamide, 5×SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5×Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42 C., with washes at 42 C. in 0.2×SSC and 0.1% SDS.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

An "exogenous" element is defined herein to mean nucleic acid sequence that is foreign to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily not found.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP 266,032 published May

4, 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler et al., *Nucl. Acids Res.,* 14: 5399–5407 [1986]). They are then purified on polyacrylamide gels.

The technique of "polymerase chain reaction," or "PCR," as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Pat. No. 4,683,195 issued Jul. 28, 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.,* 51: 263 (1987); Erlich, ed., *PCR Technology,* (Stockton Press, N.Y., 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample, comprising the use of a known nucleic acid (DNA or RNA) as a primer and utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

Suitable Methods for Practicing the Invention

Some aspects of this invention include obtaining an import, non-human antibody variable domain, producing a desired humanized antibody sequence and for humanizing an antibody gene sequence are described below. A particularly preferred method of changing a gene sequence, such as gene conversion from a non-human or consensus sequence into a humanized nucleic acid sequence, is the cassette mutagenesis procedure described in Example 1. Additionally, methods are given for obtaining and producing antibodies generally, which apply equally to native non-human antibodies as well as to humanized antibodies.

Generally, the antibodies and antibody variable domains of this invention are conventionally prepared in recombinant cell culture, as described in more detail below. Recombinant synthesis is preferred for reasons of safety and economy, but it is known to prepare peptides by chemical synthesis and to purify them from natural sources; such preparations are included within the definition of antibodies herein.

Molecular Modeling

An integral step in our approach to antibody humanization is construction of computer graphics models of the import and humanized antibodies. These models are used to determine if the six complementarity-determining regions (CDRs) can be successfully transplanted from the import framework to a human one and to determine which framework residues from the import antibody, if any, need to be incorporated into the humanized antibody in order to maintain CDR conformation. In addition, analysis of the sequences of the import and humanized antibodies and reference to the models can help to discern which framework residues are unusual and thereby might be involved in antigen binding or maintenance of proper antibody structure.

All of the humanized antibody models of this invention are based on a single three-dimensional computer graphics structure hereafter referred to as the consensus structure. This consensus structure is a key distinction from the approach of previous workers in the field, who typically begin by selecting a human antibody structure which has an amino acid sequence which is similar to the sequence of their import antibody.

The consensus structure of one embodiment of this invention was built in five steps as described below.

Step 1

Seven Fab X-ray crystal structures from the Brookhaven Protein Data Bank were used (entries 2FB4, 2RHE, 3FAB, and 1 REI which are human structures, and 2MCP, 1 FBJ, and 2HFL which are murine structures). For each structure, protein mainchain geometry and hydrogen bonding patterns were used to assign each residue to one of three secondary structure types: alpha-helix, beta-strand or other (i.e. non-helix and non-strand). The immunoglobulin residues used in superpositioning and those included in the consensus structure are shown in Table 1.

TABLE I

	Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure							
Ig[a]	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus[b]
				$V_L\kappa$ domain				
								2–11
	18–24	18–24	19–25	18–24	19–25	19–25	19–25	16–27
	32–37	34–39	39–44	32–37	32–37	32–37	33–38	33–39
								41–49
	60–66	62–68	67–72	53–66	60–65	60–65	61–66	59–77
	69–74	71–76	76–81	69–74	69–74	69–74	70–75	
	84–88	86–90	91–95	84–88	84–88	84–88	85–89	82–91
								101–105
RMS[c]		0.40	0.60	0.53	0.54	0.48	0.50	
				V_H domain				
								3–8
	18–25		18–25	18–25	18–25	18–25		17–23
	34–39		34–39	34–39	34–39	34–39		33–41
	46–52		46–52	46–52	46–52	46–52		45–51
	57–61		59–63	56–60	57–61	57–61		57–61
	68–71		70–73	67–70	68–71	68–71		66–71
	78–84		80–86	77–83	78–84	78–84		75–82
	92–99		94–101	91–98	92–99	92–99		88–94
								102–108

TABLE I-continued

Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure								
Ig[a]	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus[b]
RMS[c]			0.43	0.85	0.62	0.91		
RMS[d]	0.91		0.73	0.77	0.92			

[a]Four-letter code for Protein Data Bank file.
[b]Residue numbers for the crystal structures are taken from the Protein Data Bank files. Residue numbers for the consensus structure are according to Kabat et al.
[c]Root-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2FB4.
[d]Root-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2HFL.

Step 2

Having identified the alpha-helices and beta-strands in each of the seven structures, the structures were superimposed on one another using the INSIGHT computer program (Biosym Technologies, San Diego, Calif.) as follows: The 2FB4 structure was arbitrarily chosen as the template (or reference) structure. The 2FB4 was held fixed in space and the other six structures rotated and translated in space so that their common secondary structural elements (i.e. alpha-helices and beta-strands) were oriented such that these common elements were as close in position to one another as possible. (This superpositioning was performed using accepted mathematical formulae rather than actually physically moving the structures by hand.)

Step 3

With the seven structures thus superimposed, for each residue in the template (2FB4) Fab one calculates the distance from the template alpha-carbon atom (Cα) to the analogous Cα atom in each of the other six superimposed structures. This results in a table of Cα-Cα distances for each residue position in the sequence. Such a table is necessary in order to determine which residue positions will be included in the consensus model. Generally, is if all Cα-Cα distances for a given residue position were ≦1.0 Å, that position was included in the consensus structure. If for a given position only one Fab crystal structure was >1.0 Å, the position was included but the outlying crystal structure was not included in the next step (for this position only). In general, the seven β-strands were included in the consensus structure while some of the loops connecting the β-strands, e.g. complementarity-determining regions (CDRs), were not included in view of Cα divergence.

Step 4

For each residue which was included in the consensus structure after step 3, the average of the coordinates for individual mainchain N, Cα, C, O and Cβ atoms were calculated. Due to the averaging procedure, as well as variation in bond length, bond angle and dihedral angle among the crystal structures, this "average" structure contained some bond lengths and angles which deviated from standard geometry. For purposes of this invention, "standard geometry" is understood to include geometries commonly accepted as typical, such as the compilation of bond lengths and angles from small molecule structures in Weiner, S. J. et. al., *J. Amer. Chem. Soc.,* 106: 765–784 (1984).

Step 5

In order to correct these deviations, the final step was to subject the "average" structure to 50 cycles of energy minimization (DISCOVER program, Biosym Technologies) using the AMBER (Weiner, S. J. et. al., *J. Amer. Chem. Soc.,* 106: 765–784 (1984)) parameter set with only the Ca coordinates fixed (i.e. all other atoms are allowed to move) (energy minimization is described below). This allowed any deviant bond lengths and angles to assume a standard (chemically acceptable) geometry. See Table II.

TABLE II

Average Bond Lengths and Angles for "Average" (Before) and Energy-Minimized Consensus (After 50 Cycles) Structures

	V_LK before (Å)	V_LK after (Å)	V_H before (Å)	V_H after (Å)	Standard Geometry (Å)
N—Cα	1.459(0.012)	1.451(0.004)	1.451(0.023)	1.452(0.004)	1.449
Cα-C	1.515(0.012)	1.523(0.005)	1.507(0.033)	1.542(0.005)	1.522
O=C	1.208(0.062)	1.229(0.003)	1.160(0.177)	1.231(0.003)	1.229
C—N	1.288(0.049)	1.337(0.002)	1.282(0.065)	1.335(0.004)	1.335
Cα-Cβ	1.508(0.026)	1.530(0.002)	1.499(0.039)	1.530(0.002)	1.526
	(*)	(*)	(*)	(*)	(*)
C—N—Cα	123.5(4.2)	123.8(1.1)	125.3(4.6)	124.0(1.1)	121.9
N—Cα-C	110.0(4.0)	109.5(1.9)	110.3(2.8)	109.5(1.6)	110.1
Cα-C—N	116.6(4.0)	116.6(1.2)	117.6(5.2)	116.6(0.8)	116.6
O=C—N	123.1(4.1)	123.4(0.6)	122.2(4.9)	123.3(0.4)	122.9
N—Cα-Cβ	110.3(2.1)	109.8(0.7)	110.6(2.5)	109.8(0.6)	109.5
Cβ-Cα-C	111.4(2.4)	111.1(0.7)	111.2(2.2)	111.1(0.6)	111.1

Values in parentheses are standard deviations. Note that while some bond length and angle averages did not change appreciably after energy-minimization, the corresponding standard deviations are reduced due to deviant geometries assuming standard values after energy-minimization. Standard geometry values are from the AMBER forcefield as implemented in DISCOVER (Biosym Technologies).

The consensus structure might conceivably be dependent upon which crystal structure was chosen as the template on which the others were superimposed. As a test, the entire procedure was repeated using the crystal structure with the worst superposition versus 2FB4, i.e. the 2HFL Fab structure, as the new template (reference). The two consensus structures compare favorably (root-mean-squared deviation of 0.11 Å for all N, Cα and C atoms).

Note that the consensus structure only includes mainchain (N, Cα, C, O, Cβ atoms) coordinates for only those residues which are part of a conformation common to all seven X-ray crystal structures. For the Fab structures, these include the common β-strands (which comprise two β-sheets) and a few non-CDR loops which connect these β-strands. The consensus structure does not include CDRs or sidechains, both of which vary in their conformation among the seven structures. Also, note that the consensus structure includes only the V_L and V_H domains.

This consensus structure is used as the archetype. It is not particular to any species, and has only the basic shape without side chains. Starting with this consensus structure

the model of any import, human, or humanized Fab can be constructed as follows. Using the amino acid sequence of the particular antibody V_L and V_H domains of interest, a computer graphics program (such as INSIGHT, Biosym Technologies) is used to add sidechains and CDRs to the consensus structure. When a sidechain is added, its conformation is chosen on the basis of known Fab crystal structures (see the Background section for publications of such crystal structures) and rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193: 775–791 (1987)). The model also is constructed so that the atoms of the sidechain are positioned so as to not collide with other atoms in the Fab.

CDRs are added to the model (now having the backbone plus side chains) as follows. The size (i.e. number of amino acids) of each import CDR is compared to canonical CDR structures tabulated by Chothia et al., *Nature,* 342:877–883 (1989)) and which were derived from Fab crystals. Each CDR sequence is also reviewed for the presence or absence of certain specific amino acid residues which are identified by Chothia as structurally important: e.g. light chain residues 29 (CDR1) and 95 (CDR3), and heavy chain residues 26, 27, 29 (CDR1) and 55 (CDR2). For light chain CDR2, and heavy chain CDR3, only the size of the CDR is compared to the Chothia canonical structure. If the size and sequence (i.e. inclusion of the specific, structurally important residues as denoted by Chothia et al.) of the import CDR agrees in size and has the same structurally important residues as those of a canonical CDR, then the mainchain conformation of the import CDR in the model is taken to be the same as that of the canonical CDR. This means that the import sequence is assigned the structural configuration of the canonical CDR, which is then incorporated in the evolving model.

However, if no matching canonical CDR can be assigned for the import CDR, then one of two options can be exercised. First, using a program such as INSIGHT (Biosym Technologies), the Brookhaven Protein Data Bank can be searched for loops with a similar size to that of the import CDR and these loops can be evaluated as possible conformations for the import CDR in the model. Minimally, such loops must exhibit a conformation in which no loop atom overlaps with other protein atoms. Second, one can use available programs which calculate possible loop conformations, assuming a given loop size, using methods such as described by Bruccoleri et al., *Nature* 335: 564–568 (1988).

When all CDRs and sidechains have been added to the consensus structure to give the final model (import, human or humanized), the model is preferably subjected to energy minimization using programs which are available commercially (e.g. DISCOVER, Biosym Technologies). This technique uses complex mathematical formulae to refine the model by performing such tasks as checking that all atoms are within appropriate distances from one another and checking that bond lengths and angles are within chemically acceptable limits.

Models of a humanized, import or human antibody sequence are used in the practice of this invention to understand the impact of selected amino acid residues of the activity of the sequence being modeled. For example, such a model can show residues which may be important in antigen binding, or for maintaining the conformation of the antibody, as discussed in more detail below. Modeling can also be used to explore the potential impact of changing any amino acid residue in the antibody sequence.

Methods for Obtaining a Humanized Antibody Sequence

In the practice of this invention, the first step in humanizing an import antibody is deriving a consensus amino acid sequence into which to incorporate the import sequences. Next a model is generated for these sequences using the methods described above. In certain embodiments of this invention, the consensus human sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)), namely V_L κ subgroup I and V_H group III, and have the sequences indicated in the definitions above.

While these steps may be taken in different order, typically a structure for the candidate humanized antibody is created by transferring the at least one CDR from the non-human, import sequence into the consensus human structure, after the entire corresponding human CDR has been removed. The humanized antibody may contain human replacements of the non-human import residues at positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) or as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)). For example, huMAb4D5 contains human replacements of the muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but not as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol Bol.* 196:901–917 (1987)): V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S.

Differences between the non-human import and the human consensus framework residues are individually investigated to determine their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is desirably performed through modeling, by examination of the characteristics of the amino acids at particular locations, or determined experimentally through evaluating the effects of substitution or mutagenesis of particular amino acids.

In certain preferred embodiments of this invention, a humanized antibody is made comprising amino acid sequence of an import, non-human antibody and a human antibody, utilizing the steps of:

a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus human variable domain;

b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

1. non-covalently binds antigen directly,

2. interacts with a CDR; or

3. participates in the V_L–V_H interface; and

g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least

one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, one determines if any non-homologous residues identified in step (e) are exposed on the surface of the domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), one may retain the consensus residue.

Additionally, in certain embodiments the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71 L, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)).

In preferred embodiments, the method of this invention comprises the additional steps of searching either or both of the import, non-human and the consensus variable domain sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid residue that binds to antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antibody affinity). if the import sequence bears the glycosylation site, it is preferred to substitute that site for the corresponding residues in the consensus human sequence if the glycosylation site is reasonably expected to be important. If only the consensus sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

Another preferred embodiment of the methods of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

In certain alternate embodiments, one need not utilize the modeling and evaluation steps described above, and may instead proceed with the steps of obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus human antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus human antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:

a. (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, or

b. fin the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

Preferably, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody. If desired, one may utilize the other method steps described above for determining whether a particular amino acid residue can reasonably be expected to have undesirable effects, and remedying those effects.

If after making a humanized antibody according to the steps above and testing its activity one is not satisfied with the humanized antibody, one preferably reexamines the potential effects of the amino acids at the specific locations recited above. Additionally, it is desirable to reinvestigate any buried residues which are reasonably expected to affect the V_L–V_H interface but may not directly affect CDR conformation. It is also desirable to reevaluate the humanized antibody utilizing the steps of the methods claimed herein.

In certain embodiments of this invention, amino acid residues in the consensus human sequence are substituted for by other amino acid residues. In preferred embodiments, residues from a particular non-human import sequence are substituted, however there are circumstances where it is desired to evaluate the effects of other amino acids. For example, if after making a humanized antibody according to the steps above and testing its activity one is not satisfied with the humanized antibody, one may compare the sequences of other classes or subgroups of human antibodies, or classes or subgroups of antibodies from the particular non-human species, and determine which other amino acid side chains and amino acid residues are found at particular locations and substituting such other residues.

Antibodies

Certain aspects of this invention are directed to natural antibodies and to monoclonal antibodies, as illustrated in the Examples below and by antibody hybridomas deposited with the ATCC (as described below). Thus, the references throughout this description to the use of monoclonal antibodies are intended to include the use of natural or native antibodies as well as humanized and chimeric antibodies. As used herein, the term “antibody” includes the antibody variable domain and other separable antibody domains unless specifically excluded.

In accordance with certain aspects of this invention, antibodies to be humanized (import antibodies) are isolated from continuous hybrid cell lines formed by the fusion of antigen-primed immune lymphocytes with myeloma cells. in certain embodiments, the antibodies of this invention are obtained by routine screening. Polyclonal antibodies to an antigen generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the antigen and an adjuvant. It may be useful to conjugate the antigen or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic is anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

The route and schedule of the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for antigen titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodiesare prepared by recovering immune lymphoid cells—typically spleen cells or lymphocytes from lymph node tissue—from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, *Eur. J. Immunol.* 6:511 (1976) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture in vitro in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored. and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody. The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, Ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM as the case may be that heretofore have been used to purify these immunoglobulins from pooled plasma, e.g. ethanol or polyethylene glycol precipitation procedures. The purified antibodies are sterile filtered, and optionally are conjugated to a detectable marker such as an enzyme or spin label for use in diagnostic assays of the antigen in test samples.

While routinely rodent monoclonal antibodies are used as the source of the import antibody, the invention is not limited to any species. Additionally, techniques developed for the production of chimeric antibodies (Morrison et al., *Proc. Natl. Acad. Sci.,* 81:6851 (1984); Neuberger et al., *Nature* 312:604 (1984); Takeda et al., *Nature* 314:452 (1985)) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity (such as ability to activate human complement and mediate ADCC) can be used; such antibodies are within the scope of this invention.

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as Fab fragments) which bypass the generation of monoclonal antibodies are encompassed within the practice of this invention. One extracts antibody-specific messenger ANA molecules from immune system cells taken from an immunized animal, transcribes these into complementary DNA (cDNA), and clones the CDNA into a bacterial expressions system. One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system which contains a leader sequence that causes the expressed Fab protein to migrate to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional FAb fragments for those which bind the antigen. Such FAb fragments with specificity for the antigen are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

Amino Acid Sequence Variants

Amino acid sequence variants of the antibodies and polypeptides of this invention (referred to in herein as the target polypeptide) are prepared by introducing appropriate nucleotide changes into the DNA encoding the target polypeptide, or by in vitro synthesis of the desired target polypeptide. Such variants include, for example, humanized variants of non-human antibodies, as well as deletions from, or insertions or substitutions of, residues within particular amino acid sequences. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the target polypeptide, such as changing the number or position of glycosylation sites, altering any membrane anchoring characteristics, and/or altering the intra-cellular location of the target polypeptide by inserting, deleting, or otherwise affecting any leader sequence of the native target polypeptide.

In designing amino acid sequence variants of target polypeptides, the location of the mutation site and the nature of the mutation will depend on the target polypeptide characteristics) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1–3. In certain embodiments, these choices are guided by the methods for creating humanized sequences set forth above.

A useful method for identification of certain residues or regions of the target polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesism" as described by Cunningham and Wells (*Science,* 244: 1081–1085 [1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the

expressed target polypeptide variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. In general, the location and nature of the mutation chosen will depend upon the target polypeptide characteristic to be modified.

Amino acid sequence deletions of antibodies are generally not preferred, as maintaining the generally configuration of an antibody is believed to be necessary for its activity. Any deletions will be selected so as to preserve the structure of the target antibody.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the target polypeptide sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Examples of terminal insertions include the target polypeptide with an N-terminal methionyl residue, an artifact of the direct expression of target polypeptide in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the target polypeptide molecule to facilitate the secretion of the mature target polypeptide from recombinant host cells. Such signal sequences generally will be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or Ipp for *E. coli,* alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of the target polypeptide include the fusion to the N- or C-terminus of the target polypeptide of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli* trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published Apr. 6, 1989.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the target polypeptide molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of the target polypeptide, and sites where the amino acids found in the target polypeptide from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. Other sites for substitution are described infra, considering the effect of the substitution of the antigen binding, affinity and other characteristics of a particular target antibody.

Other sites of interest are those in which particular residues of the target polypeptides obtained from various species are identical. These positions may be important for the biological activity of the target polypeptide. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. If such substitutions result in a change in biological activity, then other changes are introduced and the products screened until the desired effect is obtained.

Substantial modifications in function or immunological identity of the target polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, lb) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gin, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of the target polypeptide that are homologous with other antibodies of the same class or subclass, or, more preferably, into the non-homologous regions of the molecule.

Any cysteine residues not involved in maintaining the proper conformation of target polypeptide also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

DNA encoding amino acid sequence variants of the target polypeptide is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the target polypeptide. A particularly preferred method of gene conversion mutagenesis is described below in Example 1. These techniques may utilized target polypeptide nucleic acid (DNA or RNA), or nucleic acid complementary to the target polypeptide nucleic acid.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of target polypeptide DNA. This technique is well known in the art as described by Adelman et al., *DNA,* 2: 183 (1983). Briefly, the target polypeptide DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the target polypeptide. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the target polypeptide DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci. USA,* 75: 5765 [1978]).

Single-stranded DNA template may also be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase 1, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for

synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the target polypeptide, and the other strand (the original template) encodes the native, unaltered sequence of the target polypeptide. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding target polypeptide variants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of target polypeptide. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, supra, the chapter by R. Higuchi, p. 61–70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1 μg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide triphosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.), and 25 pmole of each oligonucleotide primer, to a final volume of 50μl. The reaction mixture is overlayed with 35 μl mineral oil. The reaction is denatured for 5 minutes at 100° C., placed briefly on ice, and then 1 μl *Thermus aquaticus* (Taq) DNA polymerase (5 units/μl, purchased from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows: 2 min. at 55° C., then 30 sec. at 72° C., then 19 cycles of the following: 30 sec. at 94° C., 30 sec. at 55° C., and 30 sec. at 72° C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50:vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene,* 34: 315 [1985]). The starting material is the plasmid (or other vector) comprising the target polypeptide DNA to be mutated. The codon(s) in the target polypeptide DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the target polypeptide DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated target polypeptide DNA sequence.

Insertion of DNA into a Cloning Vehicle

The cDNA or genomic DNA encoding the target polypeptide is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(a) Signal Sequence Component

In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector.

The target polypeptides of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. Included within the scope of this invention are target polypeptides with any native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native target polypeptide signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin 11 leaders. For yeast secretion the native target polypeptide signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

(b) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using Bacillus species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in Bacillus genomic DNA. Transfection of Bacillus with this vector results in homologous recombination with the genome and insertion of the target polypeptide DNA. However, the recovery of genomic DNA encoding the target polypeptide is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the target polypeptide DNA.

(c) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for Bacilli.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., *J. Molec. Appl. Genet.,* 1: 327 [1982]), mycophenolic acid (Mulligan et al., *Science=:* 1422 [1980]) or hygromycin (Sugden et al., *Mol. Cell. Biol.,* 5: 410–413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the target polypeptide nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants

under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the target polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the target polypeptide are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77: 4216 [1980]. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the target polypeptide. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the target polypeptide, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., *Nature,* 22: 39 [1979]; Kingsman et al., *Gene,* 7: 141 [1979]; or Tschemper et al., *Gene,* 10: 157 [1980]). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics,* 5: 12 [1977]). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

(d) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the target polypeptide nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as that encoding the target polypeptide, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the target polypeptide by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native target polypeptide promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target polypeptide DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed target polypeptide as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang et al., *Nature,* 275: 615 [1978]; and Goeddel et al., *Nature,* 281: 544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.,* 8: 4057 [1980] and EP 36,776) and hybrid promoters such as the tao promoter (deBoer et al., *Proc. Natl. Acad. Sci, USA,* 80: 21–25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding the target polypeptide (Siebenlist et al., *Cell,* 20: 269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the target polypeptide.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.,* 255: 2073 [1980]) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.,* 2: 149 [1968]; and Holland, *Biochemistry,* 17: 4900 [1978]), such asenolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also ate advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

Target polypeptide transcription from vectors in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published Jul. 5, 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the target polypeptide sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that

also contains the SV40 viral origin of replication. Fiers et al., *Nature,* 273:113 (1978); Mulligan and Berg, *Science,* 209: 1422–1427 (1980); Pavlakis et al., *Proc. Natl. Acad. Sci. USA,* 78: 7398–7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway et al., *Gene,* 18: 355–360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Gray et al., *Nature,* 29: 503–508 (1982) on expressing cDNA encoding immune interferon in monkey cells; , Reyes et al., *Nature,* 297: 598–601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, *Proc. Natl. Acad. Sci. USA,* 79: 5166–5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells, and Gorman et al., *Proc. Natl. Aced. Sci. USA,* 79: 6777–6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(e) Enhancer Element Component

Transcription of DNA encoding the target polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10–300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins et al., *Proc. Natl. Acad. Sci. USA,* 78: 993 [1981]) and 3' (Lusky et al., *Mol. Cell Bio.* 3: 1108 [1983]) to the transcription unit, within an intron (Banerji et al., *Cell,* 33: 729 [1983]) as well as within the coding sequence itself (Osborne et al., *Mol. Cell Bio.,* 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100–270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature,* 297: 17–18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the target polypeptide DNA, but is preferably located at a site 5' from the promoter.

(f) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the target polypeptide. The 3' untranslated regions also include transcription termination sites.

Construction of suitable vectors containing one or more of the above listed components the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced is by the method of Messing et al., *Nucleic Acids Res.,* 9: 309 (1981) or by the method of Maxam et al., *Methods in Enzymology* 65: 499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the target polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of the target polypeptide that have target polypeptide-like activity.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the target polypeptide in recombinant vertebrate cell culture are described in Gething et al., *Nature,* 293: 620–625 [1981]; Mantei et al., *Nature,* 281: 40–46 [1979]; Levinson et al.,; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of the target polypeptide is pRK5 (EP pub. no. 307,247) or pSVI6B.

Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli* , Bacilli such as *B. subtilis*, Pseudomonas species such as *P. aeruginosa, Salmonella typhimurium,* or *Serratia marcescans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, in vitro methods of cloning, e.g. PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for target polypeptide-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* [Beach and Nurse, *Nature,* 290: 140 (1981); EP 139,383 published May 2, 1985], Kluyveromyces hosts (U.S. Pat. No. 4,943,529) such as, e.g., *K. lactis* [Louvencourt et al., *J. Bacteriol.,* 737 (1983)], *K. fragilis, K. bulgaricus, K. thermotolerans,* and *K. marxianus,* yarrowia [EP 402,226], *Pichia pastoris* [EP 183,070; Sreekrishna et al., *J. Basic Microbiol.,* 28: 265–278 (1988)], Candida, *Trichoderma reesia* [EP 244,2341], *Neurospora crassa* [Case et al., *Proc. Natl. Acad. Sci. USA,* 76: 5259–5263 (1979)], and filamentous fungi such as, e.g, Neurospora, Penicillium, Tolypocladium [WO 91/00357 published Jan. 10, 1991], and Aspergillus hosts such as *A. nidulans* [Ballance et al., *Biochem. Biophys. Res. Commun.* 112: 284–289 (1983); Tilburn et al., *Gene,* 26: 205–221 (1983); Yelton et al., *Proc. Natl. Acad. Sci. USA,* 81: 1470–1474 (1984)] and *A. niger* [Kelly and Hynes, *EMBO J.,* 4: 475–479 (1985)].

Suitable host cells for the expression of glycosylated target polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified. See, e.g., Luckow et al., *Bio/Technologvy* 6: 47–55 (1988); Miller et al., in *Genetic Engineering* Setlow, J. K. et a., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277–279; and Maeda et al., *Nature,* 315: 592–594 (1985). A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cutures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens,* which has been previously manipulated to contain the target polypeptide DNA. During incubation of the plant cell culture with *A. tumefaciens,* the DNA encoding target polypeptide is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the target polypeptide DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al.,*J. Mol. Appl. Gen.,* 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published Jun. 21, 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.,* 36: 59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77: 4216 [1980]); mouse sertoli cells (TM4, Mather, *Biol. Reprod.,* 23: 243–251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HS 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.,* 383: 44–68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., supra, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., *Gene,* 23: 315 (1983) and WO 89/05859 published Jun. 29, 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30–16.37 of Sambrook et al., supra, is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued Aug. 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.,* 130: 946 (1977) and Hsiao et al., *Proc. Natl. Acad. Sci. (USA),* 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

Culturina the Host Cells

Prokaryotic cells used to produce the target polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce the target polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.,* 58: 44 (1979), Barnes and Sato, *Anal. Biochem.* 102: 255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. Re. 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in in vitro culture as well as cells that are within a host animal.

It is further envisioned that the target polypeptides of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the target polypeptide currently in use in the field.

For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired target polypeptide. The control element does not encode the target polypeptide of this invention, but the DNA is present in the host cell genome. One next screens for cells making the target polypeptide of this invention, or increased or decreased levels of expression, as desired.

Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA,* 77: 5201–5205 [1980]), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu et al., *Am. J. Clin. Path.,* 75: 734–738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native target polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

Purification of the Target Polypeptide

The target polypeptide preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When the target polypeptide is expressed in a recombinant cell other than one of human origin, the target polypeptide is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the target polypeptide from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the target polypeptide. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The target polypeptide may then be purified from the soluble protein fraction and from the membrane fraction of the culture lysate, depending on whether the target polypeptide is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Target polypeptide variants in which residues have been deleted, inserted or substituted are recovered in the same fashion, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a target polypeptide fusion with another protein or polypeptide, e.g. a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen (or containing antigen, where the target polypeptide is an antibody) can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-target polypeptide column can be employed to absorb the target polypeptide variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native target polypeptide may require modification to account for changes in the character of the target polypeptide or its variants upon expression in recombinant cell culture.

Covalent Modifications of Target Polypeptides

Covalent modifications of target polypeptides are included within the scope of this invention. One type of covalent modification included within the scope of this invention is a target polypeptide fragment. Target polypeptide fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length target polypeptide or variant target polypeptide. Other types of covalent modifications of the target polypeptide or fragments thereof are introduced into the molecule by reacting specific amino acid residues of the target polypeptide or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides,3-nitro-2-pyridyl disulfide, methyl2-pyridyldisulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5–7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal;

chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in so introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'—N═C═N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking target polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-target polypeptide antibodies, and vice versa. Commonly used crosslinking agents include, e.g., 1,1-bis (diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis (succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modification include hydroxylation of proline and lysine, phophorylation of hydroxyl groups of seryl or threonyl resides, methylation of the α-amino groups of lysine, arginine, and histidine side chains, (T. E. Creighton, *Protein: Structure and Molecular Properties*, W. H. Freeman & Co., San Francisco, pp. 79–86 [1983]), acetylation of the N-terminal amine, and amidaatioon of any C-terminal carboxyl group.

Another type of covalent modification of the target polypeptide included within the scope of this invention comprises altering the native glucosylatuion pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in the native target polypeptide, and/or adding one or more glycosylation sites that are not present in the native target polypeptide.

Gylcosylation of polypeptides is typically either N-linked or O-linked refers to the attachment of the carbonhydrate moiety to the side chain of an asparagine reisdue. The tri-peptide sequences asparagine-X-resine and asparagine-X-threonine, where X is any aminoe acid except proline, are the recoginition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgactosamine, galactose, or xylose, to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the target polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of hte above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or theonine resides to the native target polypeptide sequence (for O-linked glycosylation sites). For ease, the target polypeptide amino acid sequences is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the target polypeptide at preselected bases such that condons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of Target Polypeptide".

Another means of increasing the number of carbohydrate moieties on the target polypeptide is by chemical or enzymatic coupling glycosides to the polypeptides. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the couple mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston (*CRC Crit. Rev. Biochem., pp.* 259–306 [1981]).

Removal of carbohydrate moieties present on the native target polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin et al. (*Arch. Biochem. Biophys.,* 259:52 [1987]) and by Edge et al. (*Anal. Biochem.,* 118:131 [1981]). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. (*Meth. Enzymol.* 138:350 [1987]).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al. (*J. Biol. Chem.,* 257:3105 [1982]). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of the target polypeptide comprises linking the target polypeptide to various nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the

manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The target polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate]microcapsules, respectively), in colloidal drug deliverysystems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Reminaton's Pharmaceutical Sciences,* 16th edition, Osol, A., Ed., (1980).

Target polypeptide preparations are also useful in generating antibodies, for screening for binding partners, as standards in assays for the target polypeptide (e.g. by labeling the target polypeptide for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant target polypeptide, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. For example, a change in the immunological character of the target polypeptide molecule, such as affinity for a given antigen or antibody, is measured by a competitive-type immunoassay. The variant is assayed for changes in the suppression or enhancement of its activity by comparison to the activity observed for the target polypeptide in the same assay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability in recombinant cell culture or in plasma, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

Diagnostic and Related Uses of the Antibodies

The antibodies of this invention are useful in diagnostic assays for antigen expression in specific cells or tissues. The antibodies are detectably labeled and/or are immobilized on an insoluble matrix.

The antibodies of this invention find further use for the affinity purification of the antigen from recombinant cell culture or natural sources. Suitable diagnostic assays for the antigen and its antibodies depend on the particular antigen or antibody. Generally, such assays include competitive and sandwich assays, and steric inhibition assays. Competitive and sandwich methods employ a phase-separation step as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of the antigen and for substances that bind the antigen, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors, or antigens.

Analytical methods for the antigen or its antibodies all use one or more of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner and steric conjugates. The labeled reagents also are known as "tracers."

The label used (and this is also useful to label antigen nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotintavidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalenily to proteins or polypeptides. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. No. 3,940,475 (fluorimetry) and U.S. Pat. No. 3,645,090 (enzymes); Hunter et al., *Nature,* 144: 945 (1962); David et al., *Biochemistry,* 13: 1014–1021 (1974); Pain et al., *J. Immunol. Methods,* 40: 219–230 (1981); and Nygren, *J. Histochem. and Cytochem.,* 30: 407–412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase.

The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan et al., "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in *Methods in in Enzymology*, ed. J. J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, N.Y., 1981), pp. 147–166. Such bonding methods are suitable for use with the antibodies and polypeptides of this invention.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. Pat. No. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analogue afterward, e.g., by immunoprecipitation.

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-

response curves with known amounts of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared and used such that when anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, the antigen or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with antibody so that binding of the antibody inhibits or potentiates the enzyme activity of the label. This method per se is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in fluorescence when the hapten is a fluorophore.

Sandwich assays particularly are useful for the determination of antigen or antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled binding partner. A sequential sandwich assay using an anti-antigen monoclonal antibody as one antibody and a polyclonal anti-antigen antibody as the other is useful in testing samples for particular antigen activity.

The foregoing are merely exemplary diagnostic assays for the import and humanized antibodies of this invention. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described above.

Immunotoxins

This invention is also directed to immunochemical derivatives of the antibodies of this invention such as immunotoxins (conjugates of the antibody and a cytotoxic moiety). Antibodies which carry the appropriate effector functions, such as with their constant domains, are also used to induce lysis through the natural complement process, and to interact with antibody dependent cytotoxic cells normally present.

For example, purified, sterile filtered antibodies are optionally conjugated to a cytotoxin such as ricin for use in AIDS therapy. U.S. patent application Ser. No. 07/350,895 illustrates methods for making and using immunotoxins for the treatment of HIV infection. The methods of this invention, for example, are suitable for obtaining humanized antibodies for use as immunotoxins for use in AIDS therapy.

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal, plant or animal origin, or an enzymatically active fragment of such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. In another embodiment, the antibodies are conjugated to small molecule anticancer drugs such as cis-platin or 5FU. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

Immunotoxins can be made in a variety of ways, as discussed herein. Commonly known crosslinking reagents can be used to yield stable conjugates.

Advantageously, monoclonal antibodies specifically binding the domain of the antigen which is exposed on the infected cell surface, are conjugated to ricin A chain. Most advantageously the ricin A chain is deglycosylated and produced through recombinant means. An advantageous method of making the ricin immunotoxin is described in Vitetta et al., *Science* 238:1098 (1987).

When used to kill infected human cells in vitro for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for in vitro use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques.

Cytotoxic radiopharmaceuticals for treating infected cells may be made by conjugating radioactive isotopes (e.g. I, Y, Pr) to the antibodies. Advantageously alpha particle-emitting isotopes are used. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

In a preferred embodiment, ricin A chain is deglycosylated or produced without oligosaccharides, to decrease its clearance by irrelevant clearance mechanisms (e.g., the liver). In another embodiment, whole ricin (A chain plus B chain) is conjugated to antibody if the galactose binding property of B-chain can be blocked ("blocked ricin").

In a further embodiment toxin-conjugates are made with Fab or $F(ab')_2$ fragments. Because of their relatively small size these fragments can better penetrate tissue to reach infected cells.

In another embodiment, fusogenic liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding the particular antigen.

Antibody Dependent Cellular Cytotoxicity

Certain aspects of this invention involve antibodies which are (a) directed against a particular antigen and (b) belong to a subclass or isotype that is capable of mediating the lysis of cells to which the antibody molecule binds. More specifically, these antibodies should belong to a subclass or isotype that, upon complexing with cell surface proteins, activates serum complement and/or mediates antibody dependent cellular cytotoxicity (ADCC) by activating effector cells such as natural killer cells or macrophages.

Biological activity of antibodies is known to be determined, to a large extent, by the constant domains or Fc region of the antibody molecule (Uananue and Benacerraf,

Textbook of Immunology, 2nd Edition, Williams & Wilkins, p. 218 (1984)). This includes their ability to activate complement and to mediate antibody-dependent cellular cytotoxicity (ADCC) as effected by leukocytes. Antibodies of different classes and subclasses differ in this respect, as do antibodies from the same subclass but different species; according to the present invention, antibodies of those classes having the desired biological activity are prepared. Preparation of these antibodies involves the selection of antibody constant domains are their incorporation in the humanized antibody by known technique. For example, mouse immunoglobulins of the IgG3 and lgG2a class are capable of activating serum complement upon binding to the target cells which express the cognate antigen, and therefore humanized antibodies which incorporate IgG3 and lgG2a effector functions are desirable for certain therapeutic applications.

In general, mouse antibodies of the IgG2a and IgG3 subclass and occasionally IgG1 can mediate ADCC, and antibodies of the IgG3, IgG2a, and IgM subclasses bind and activate serum complement. Complement activation generally requires the binding of at least two IgG molecules in close proximity on the target cell. However, the binding of only one IgM molecule activates serum complement.

The ability of any particular antibody to mediate lysis of the target cell by complement activation and/or AOCC can be assayed. The cells of interest are grown and labeled in vitro; the antibody is added to the cell culture in combination with either serum complement or immune cells which may be activated by the antigen antibody complexes. Cytolysis of the target cells is detected by the release of label from the lysed cells. In fact, antibodies can be screened using the patient's own serum as a source of complement and/or immune cells. The antibody that is capable of activating complement or mediating ADCC in the in vitro test can then be used therapeutically in that particular patient.

This invention specifically encompasses consensus Fc antibody domains prepared and used according to the teachings of this invention.

Therapeutic and Other Uses of the Antibodies

When used in vivo for therapy, the antibodies of the subject invention are administered to the patient in therapeutically effective amounts (i.e. amounts that have desired therapeutic effect). They will normally be administered parenterally. The dose and dosage regimen will depend upon the degree of the infection, the characteristics of the particular antibody or immunotoxin used, e.g., its therapeutic index, the patient, and the patient's history. Advantageously the antibody or immunotoxin is administered continuously over a period of 1–2 weeks, intravenously to treat cells in the vasculature and subcutaneously and intraperitoneally to treat regional lymph nodes. Optionally, the administration is made during the course of adjunct therapy such as combined cycles of radiation, chemotherapeutic treatment, or administration of tumor necrosis factor, interferon or other cytoprotective or immunomodulatory agent.

For parenteral administration the antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic, and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate can also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The antibodies will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

Use of IgM antibodies may be preferred for certain applications, however IgG molecules by being smaller may be more able than IgM molecules to localize to certain types of infected cells.

There is evidence that complement activation in vivo-leads to a variety of biological effects, including the induction of an inflammatory response and the activation of macrophages (Uananue and Benecerraf, *Textbook of Immunology,* 2nd Edition, Williams & Wilkins, p. 218 (1984)). The increased vasodilation accompanying inflammation may increase the ability of various agents to localize in infected cells. Therefore, antigen-antibody combinations of the type specified by this invention can be used therapeutically in many ways. Additionally, purified antigens (Hakomori, *Ann. Rev. Immunol.* 2:103 (1984)) or anti-idiotypic antibodies (Nepom et al., *Proc. Natl. Acad. Sci.* 81:2864 (1985); Koprowski et al., *Proc. Natl. Acad. Sci.* 81:216 (1984)) relating to such antigens could be used to induce an active immune response in human patients. Such a response includes the formation of antibodies capable of activating human complement and mediating ADCC and by such mechanisms cause infected cell destruction.

Optionally, the antibodies of this invention are useful in passively immunizing patients, as exemplified by the administration of humanized anti-HIV antibodies.

The antibody compositions used in therapy are formulated and dosages established in a fashion consistent with good medical practice taking into account the disorder to be treated, the condition of the individual patient, the site of delivery of the composition, the method of administration and other factors known to practitioners. The antibody compositions are prepared for administration according to the description of preparation of polypeptides for administration, infra.

Deposit of Materials

As described above, cultures of the muMAb4D5 have been deposited with the American Type Culture Collection, 10801 University Blvd., Mauassas, Va., USA (ATCC).

This deposit was made under the provisions of the Budapest Treaty on the international Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of the deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures' availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.12 with particular reference to 886 OG 638).

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed

when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the constructs deposited, since the deposited embodiments are intended to illustrate only certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention.

EXAMPLES

Example 1

Humanization of muMAb4D5

Here we report the chimerization of muMAb4D5 (chMAb4D5) and the rapid and simultaneous humanization of heavy (V_H) and light (V_L) chain variable region genes using a novel "gene conversion mutagenesis" strategy. Eight humanized variants (huMAb4D5) were constructed to probe the importance of several FR residues identified by our molecular modeling or previously proposed to be critical to the conformation of particular CDRs (see Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987); Chothia, C. et al., *Nature* 342:877–883 (1989); Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)). Efficient transient expression of humanized variants in non-myeloma cells allowed us to rapidly investigate the relationship between binding affinity for $p185^{HER2}$ ECD and anti-proliferative activity against $p185^{HER2}$ overexpressing carcinoma cells.

Materials and Methods

Cloning of Variable Region Genes. The muMAb4D5 V_H and V_L genes were isolated by polymerase chain reaction (PCR) amplification of mRNA from the corresponding hybridoma (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) as described by Orlandi et al. (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833–3837 (1989)). Amino terminal sequencing of muMAb4D5 V_L and V_H was used to design the sense strand PCR primers, whereas the anti-sense PCR primers were based upon consensus sequences of murine framework residues (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833–3837 (1989); Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) incorporating restriction sites for directional cloning shown by underlining and listed after the sequences: V_L sense, 5'-TCC GATATCCAGCTGACCCAGTCTCCA-3' (SEQ. ID NO. 7), EcoRV; V_L anti-sense, 5'-GTTTGATCTCCAGCTT GGTACCHSCDCCGAA-3' (SEQ. ID NO. 8), Asp718; V_H sense, 5'-AGGTSMARCTGCAGSAGTCWGG-3' (SEQ. ID NO. 9), PstI and V_H anti-sense, 5'-TGAGGAGAC GGTGACCGTGGTCCCTTGGCCCCAG-3' (SEQ. ID. NO. 10), BstEII; where H=A or C or T, S=C or G, D=A or G or T, M=A or C, R=A or G and W=A or T. The PCR products were cloned into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3–11 (1987)) and five clones for each variable domain sequenced by the dideoxy method (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463–5467 (1977)).

Molecular Modelling. Models for muMAb4D5 V_H and V_L domains were constructed separately from consensus coordinates based upon seven Fab structures from the Brookhaven protein data bank (entries 1FB4, 2RHE, 2MCP, 3FAB, 1FBJ, 2HFL and 1REI). The Fab fragment KOL (Marquart, M. et al., *J. Mol. Biol.* 141:369–391 (1980)) was first chosen as a template for V_L and V_H domains and additional structures were then superimposed upon this structure using their main chain atom coordinates (INSIGHT program, Siosym Technologies). The distance from the template Cα to the analogous Cα in each of the superimposed structures was calculated for each residue position. If all (or nearly all) Cα—Cα distances for a given residue were ≦1 Å, then that position was included in the consensus structure. In most cases the β-sheet framework residues satisfied these criteria whereas the CDR loops did not. For each of these selected residues the average coordinates for individual N, Cα, C, O and Cβ atoms were calculated and then corrected for resultant deviations from non-standard bond geometry by 50 cycles of energy minimization using the DISCOVER program (Biosym Technologies) with the AMBER forcefield (Weiner, S. J. et al., *J. Amer. Chem. Soc.* 106:765–784 (1984)) and Cα coordinates fixed. The side chains of highly conserved residues, such as the disulfide-bridged cysteine residues, were then incorporated into the resultant consensus structure. Next the sequences of muMAb4D5 V_L and V_H were incorporated starting with the CDR residues and using the tabulations of CDR conformations from Chothia et al. (Chothia, C. et al., *Nature* 342:877–883 (1989)) as a guide. Side-chain conformations were chosen on the basis of Fab crystal structures, rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193:775–791 (1987)) and packing considerations. Since V_H-COR3 could not be assigned a definite backbone conformation from these criteria, two models were created from a search of similar sized loops using the INSIGHT program. A third model was derived using packing and solvent exposure considerations. Each model was then subjected to 5000 cycles of energy minimization.

In humanizing muMAb4D5, consensus human sequences were first derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)), namely V_L κ subgroup I and V_H group III, and a molecular model generated for these sequences using the methods described above. A structure for huMAb4D5 was created by transferring the CDRs from the muMAb4D5 model into the consensus human structure. All huMAb4D5 variants contain human replacements of muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but notas defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)):

V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S. Differences between muMAb4D5 and the human consensus framework residues (FIG. **1**) were individually modeled to investigate their possible influence on CDR conformation and/or binding to the $p185^{HER2}$ ECD.

Construction of Chimeric Genes. Genes encoding chMAb4D5 light and heavy chains were separately assembled in previously described phagemid vectors containing ihe human cytomegalovirus enhancer and promoter, a 5' intron and SV40 polyadenylation signal (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3–10 (1990)). Briefly, gene segments encoding muMAb4D5 V_L (FIG. **1**A) and REI human κ_1 light chain C_L (Palm, W. & Hilschmann, N., *Z. Physiol. Chem.* 356:167–191 (1975)) were precisely joined as were genes for muMAb4D5 V_H (FIG. **1**B) and human γ1 constant region (Capon, D. J. et al., *Nature* 337:525–531 (1989)) by simple subcloning (Boyle, A., in *Current Protocols in Molecular Biology,* Chapter 3 (F. A. Ausubel et al., eds., Greene Publishing & Wiley-Interscience, New York, 1990)) and site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The γ1 isotype was chosen as it has been found to be the preferred human isotype for supporting ADCC and complement dependent cytotoxicity using matched sets of chimeric (Brüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987)) or humanized antibodies (Riechmann, L. et al., *Nature* 332:323–327 (1988)). The PCR-generated V_L and V_H fragments (FIG. **1**) were subsequently mutagenized so that they faithfully represent the sequence of muMAb4D5 determined at the protein level: V_H Q1E, V_L V_{104}L and T109A (variants are denoted by the amino acid residue and number followed by the replacement amino acid). The human γ1 constant regions are identical to those reported by Ellison et al. (Ellison, J. W. et al., *Nucleic Acids Res.* 13:4071–4079 (1982)) except for the mutations E359D and M361L (Eu numbering, as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) which we installed to convert the antibody from the naturally rare A allotype to the much more common non-A allotype (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)). This was an attempt to reduce the risk of anti-allotype antibodies interfering with therapy.

Construction of Humanized Genes. Genes encoding chMAb4D5 light chain and heavy chain Fd fragment (V_H and C_H1 domains) were subcloned together into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3–11 (1987)) to create pAK1 and simultaneously humanized in a single step (FIG. **2**). Briefly, sets of 6 contiguous oligonucleotides were designed to humanize V_H and V_L (FIG. **1**). These oligonucleotides are 28 to 83 nucleotides in length, contain zero to 19 mismatches to the murine antibody template and are constrained to have 8 or 9 perfectly matched residues at each end to promote efficient annealing and ligation of adjacent oligonucleotides. The sets of V_H and V_L humanization oligonucleotides (5 pmol each) were phosphorylated with either ATP or γ-^{32}P-ATP (Carter, P. *Methods Enzymol.* 154: 382–403 (1987)) and separately annealed with 3.7 pmol of pAK1 template in 40 μl 10 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ by cooling from 100° C. to room temperature over ~30 min. The annealed oligonucleotides were joined by incubation with T4 DNA ligase (12 units; New England Biolabs) in the presence of 2 μl 5 mM ATP and 2 μl 0.1 M DTT for 10 min at 14° C. After electrophoresis on a 6% acrylamide sequencing gel the assembled oligonucleotides were located by autoradiography and recovered by electroelution. The assembled oligonucleotides (~0.3 pmol each) were simultaneously annealed to 0.15 pmol single-stranded deoxyuridine-containing pAK1 prepared according to Kunkel et al. (Kunkel, T. A. et al., *Methods Enzymol.* 154:367–382 (1987)) in 10 μl 40 mM Tris-HCl (pH 7.5) and 16 mM $MgCl_2$ as above. Heteroduplex DNA was constructed by extending the primers with T7 DNA polymerase and transformed into *E. coli* BMH 71–18 mutL as previously described (Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991)). The resultant phagemid DNA pool was enriched first for huV_L by restriction purification using XhoI and then for huV_H by restriction selection using StuI as described in Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991); and in Wells, J. A. et al., *Phil. Trans. R. Soc. Lond., A* 317:415–423 (1986). Resultant clones containing both huV_L and huV_H genes were identified by nucleotide sequencing (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463–5467 (1977)) and designated pAK2. Additional humanized variants were generated by site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991)). The muMAb4D5 V_L and V_H gene segments in the transient expression vectors described above were then precisely replaced with their humanized versions.

Expression and Purification of MAb4D5 Variants. Appropriate MAb4D5 light and heavy chain cDNA expression vectors were co-transfected into an adenovirus transformed human embryonic kidney cell line, 293 (Graham, F. L. et al., *J. Gen. Virol.* 36:59–72 (1977)) using a high efficiency procedure (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3–10 (1990); Gorman, C., in *DNA Cloning,* vol II, pp 143–190 (D. M. Glover, ed., IRL Press, Oxford, UK 1985)). Media were harvested daily for up to 5 days and the cells re-fed with serum free media. Antibodies were recovered from the media and affinity purified on protein A sepharose CL-4B (Pharmacia) as described by the manufacturer. The eluted antibody was buffer-exchanged into phosphate-buffered saline by G25 gel filtration, concentrated by ultrafiltration (Centriprep-30 or Centricon-100, Amicon), sterile-filtered (Millex-GV, Millipore) and stored at 4° C. The concentration of antibody was determined by using both total immunoglobulin and antigen binding ELISAs. The standard used was huMAb4D5-5, whose concentration had been determined by amino acid composition analysis.

Cell Proliferation Assay. The effect of MAb4D5 variants upon proliferation of the human mammary adenocarcinoma cell line, SK-BR-3, was investigated as previously described (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) using saturating MAb4D5 concentrations.

Affinity Measurements. The antigen binding affinity of MAb4D5 variants was determined using a secreted form of the $p185^{HER2}$ ECD prepared as described in Fendly, B. M. et al., *J. Biol. Resp. Mod.* 9:449–455 (1990). Briefly, antibody and $p185^{HER2}$ ECD were incubated in solution until equilibrium was found to be reached. The concentration of free antibody was then determined by ELISA using immobilized $p185^{HER2}$ ECD and used to calculate affinity (K_d) according to Friguet et al. (Friguet, B. et al., *J. Immunol. Methods* 77:305–319 (1985)).

Results

Humanization of muMAb4D5. The muMAb4D5 V_L and V_H gene segments were first cloned by PCR and sequenced (FIG. **1**). The variable genes were then simultaneously humanized by gene conversion mutagenesis using preassembled oligonucleotides (FIG. **2**). A 311-mer oligonucle-

otide containing 39 mismatches to the template directed 24 simultaneous amino acid changes required to humanize muMAb4D5 V_L. Humanization of muMAb4D5 V_H required 32 amino acid changes which were installed with a 361-mer containing 59 mismatches to the muMAb4D5 template. Two out of 8 clones sequenced precisely encode huMAb4D5-5, although one of these clones contained a single nucleotide imperfection. The 6 other clones were essentially humanized but contained a small number of errors: <3 nucleotide changes and <1 single nucleotide deletion per kilobase. Additional humanized variants (Table 3) were constructed by site-directed mutagenesis of huMAb4D5-5.

Expression levels of huMAb4D5 variants were in the range of 7 to 15 μg/ml as judged by ELISA using immobilized $p185^{HER2}$ ECD. Successive harvests of five 10 cm plates allowed 200 μg to 500 mg of each variant to be produced in a week. Antibodies affinity purified on protein A gave a single band on a Coomassie blue stained SDS polyacrylamide gel of mobility consistent with the expected M_r of ~150 kDa. Electrophoresis under reducing conditions gave 2 bands consistent with the expected M_r of free heavy (48 kDa) and light (23 kDa) chains (not shown). Amino terminal sequence analysis (10-cycles) gave the mixed sequence expected (see FIG. **1**) from an equimolar combination of light and heavy chains (not shown).

huMAb4D5 Variants. In general, the FR residues were chosen from consensus human sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) and CDR residues from muMAb4D5. Additional variants were constructed by replacing selected human residues in huMAb4D5-1 with their muMAb4D5 counterparts. These are V_H residues 71, 73, 78, 93 plus 102 and V_L residues 55 plus 66 identified by our molecular modeling. V_H residue 71 has previously been proposed by others (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) to be critical to the conformation of V_H-CDR2. Amino acid sequence differences between huMAb4D5 variant molecules are shown in Table 3, together with their $p185^{HER2}$ ECD binding affinity and maximal anti-proliferative activities against SK-BR-3 cells. Very similar K_d values were obtained for binding of MAb4D5 variants to either SK-BR-3 cells or to $p185^{HER2}$ ECD (Table 3). However, K_d estimates derived from binding of MAb4D5 variants to $p185^{HER2}$ ECD were more reproducible with smaller standard errors and consumed much smaller quantities of antibody than binding measurements with whole cells.

The most potent humanized variant designed by molecular modeling, huMAb4D5-8, contains 5 FR residues from muMAb4D5. This antibody binds the $p185^{HER2}$ ECD 3-fold more tightly than does muMAb4D5 itself (Table 3) and has comparable anti-proliferative activity with SK-BR-3 cells (FIG. **3**). In contrast, huMAb4D5-1 is the most humanized but least potent muMAb4D5 variant, created by simply installing the muMAb4D5 CDRs into the consensus human sequences. huMAb4D5-1 binds the $p185^{HER2}$ ECD 80-fold less tightly than does the murine antibody and has no detectable anti-proliferative activity at the highest antibody concentration investigated (16 μg/ml).

The anti-proliferative activity of huMAb4D5 variants against $p185^{HER2}$ overexpressing SK-BR-3 cells is not simply correlated with their binding affinity for the $p185^{HER2}$ ECD. For example, installation of three murine residues into the V_H domain of huMAb4D5-2 (D73T, L78A and A93S) to create huMAb4D5-3 does not change the antigen binding affinity but does confer significant anti-proliferative activity (Table 3).

The importance of V_H residue 71 (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) is supported by the observed 5-fold increase in affinity for $p185^{HER2}$ ECD on replacement of R71 in huMAb4D5-1 with the corresponding murine residue, alanine (huMAb4D5-2). In contrast, replacing V_H L78 in huMAb4D5-4 with the murine residue, alanine (huMAb4D5-5), does not significantly change the affinity for the $p185^{HER2}$ ECD or change anti-proliferative activity, suggesting that residue 78 is not of critical functional significance to huMAb4D5 and its ability to interact properly with the extracellular domain of $p185^{HER2}$.

V_L residue 66 is usually a glycine in human and murine κ chain sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but an arginine occupies this position in the muMAb4D5 κ light chain. The side chain of residue 66 is likely to affect the conformation of V_L-CDR1 and V_L-CDR2 and the hairpin turn at 68–69 (FIG. **4**). Consistent with the importance of this residue, the mutation V_L G66R (huMAb4D5-3→huMAb4D5-5) increases the affinity for the $p185^{HER2}$ ECD by 4-fold with a concomitant increase in anti-proliferative activity.

From molecular modeling it appears that the tyrosyl side chain of muMAb4D5 V_L residue 55 may either stabilize the conformation of V_H-CDR3 or provide an interaction at the V_L–V_H interface. The latter function may be dependent upon the presence of V_H Y102. In the context of huMAb4D5-5 the mutations V_L E55Y (huMAb4D5-6) and V_H V102Y (huMAb4D5-7) individually increase the affinity for $p185^{HER2}$ ECD by 5-fold and 2-fold respectively, whereas together (huMAb4D5-8) they increase the affinity by 11-fold. This is consistent with either proposed role of V_L Y55 and V_H Y102.

Secondary Immune Function of huMAb4D5-8. MuMAb4D5 inhibits the growth of human breast tumor cells which overexpress $p185^{HER2}$ (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165–1172 (1989)). The antibody, however, does not offer the possibility of direct tumor cytotoxic effects. This possibility does arise in huMAb4D5-8 as a result of its high affinity (Kd_d=0.1 μM) and its human IgG_1 subtype. Table 4 compares the ADCC mediated by huMAb4D5-8 with muMAb4D5 on a normal lung epithelial cell line, WI-38, which expresses a low level of $p185^{HER2}$ and on SK-BR-3, which expresses a high level of $p185^{HER2}$. The results demonstrate that: (1) huMAb4D5 has a greatly enhanced ability to carry out ADCC as compared with its murine parent; and (2) that this activity may be selective for cell types which overexpress $p185^{HER2}$.

Discussion

MuMAb4D5 is potentially useful for human therapy since it is cytostatic towards human breast and ovarian tumor lines overexpressing the HER2-encoded $p185^{HER2}$ receptor-like tyrosine kinase. Since both breast and ovarian carcinomas are chronic diseases it is anticipated that the optimal MAb4D5 variant molecule for therapy will have low immunogenicity and will be cytotoxic rather than solely cytostatic in effect. Humanization of muMAb4D5 should accomplish these goals. We have identified 5 different huMAb4D5 variants which bind tightly to $p185^{HER2}$ ECD ($K_d \leqq 1$ nM) and which have significant anti-proliferative activity (Table 3). Furthermore huMAb4D5-8 but not muMAb4D5 mediates ADCC against human tumor cell lines overexpressing $p185^{HER2}$ in the presence of human effector cells (Table 4) as anticipated for a human γ1 isotype (Brcüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987); Riechmann, L. et al., *Nature* 332:323–327 (1988)).

Rapid humanization of huMAb4D5 was facilitated by the gene conversion mutagenesis strategy developed here using long preassembled oligonucleotides. This method requires less than half the amount of synthetic DNA as does total gene synthesis and does not require convenient restriction sites in the target DNA. Our method appears to be simpler and more reliable than a variant protocol recently reported (Rostapshov, V. M. et al., *FEBS Lett.* 249: 379–382 (1989)). Transient expression of huMAb4D5 in human embryonic kidney 293 cells permitted the isolation of a few hundred micrograms of huMAb4D5 variants for rapid characterization by growth inhibition and antigen binding affinity assays. Furthermore, different combinations of light and heavy chain were readily tested by co-transfection of corresponding cDNA expression vectors.

The crucial role of molecular modeling in the humanization of muMAb4D5 is illustrated by the designed variant huMAb4D5-8 which binds the p185^{HER2} ECD 250-fold more tightly than the simple CDR loop swap variant, huMAb4D5-1. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., *Nature* 332:323–327 (1988); Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)). Here we have extended this earlier work by others with a designed humanized antibody which binds its antigen 3-fold more tightly than the parent rodent antibody. While this result is gratifying, assessment of the success of the molecular modeling must await the outcome of X-ray structure determination. From analysis of huMAb4D5 variants (Table 3) it is apparent that their anti-proliferative activity is not a simple function of their binding affinity for p185^{HER2} ECD. For example the huMAb4D5–8 variant binds p185^{HER2} 3-fold more tightly than muMAb4D5 but the humanized variant is slightly less potent in blocking the proliferation of SK-SR-3 cells. Additional huMAb4D5 variants are currently being constructed in an attempt to identify residues triggering the anti-proliferative activity and in an attempt to enhance this activity.

In addition to retaining tight receptor binding and the ability to inhibit cell growth, the huMAb4D5-8 also confers a secondary immune function (ADCC). This allows for direct cytotoxic activity of the humanized molecule in the presence of human effector cells. The apparent selectivity of the cytotoxic activity for cell types which overexpress p185^{HER2} allows for the evolution of a straightforward clinic approach to those human cancers characterized by overexpression of the HER2 protooncogene.

TABLE 3

p185^{HER2} ECD binding affinity and anti-proliferative activities of MAb4D5 variants

	V_H Residue*					V_L Residue*			
MAb4D5 cell Variant proliferation‡	71 FR3	73 FR3	78 FR3	93 FR3	102 CDR3	55 CDR2	56 FR3	K_d† nM	Relative
huMAb4D5-1	R	D	L	A	V	E	G	25	102
huMAb4D5-2	Ala	D	L	A	V	E	G	4.7	101
huMAb4D5-3	Ala	Thr	Ala	Ser	V	E	G	4.4	66
huMAb4D5-4	Ala	Thr	L	Ser	V	E	Arg	0.82	56
huMAb4D5-5	Ala	Thre	Ala	Ser	V	E	Arg	1.1	48
huMAb4D5-6	Ala	Thr	Ala	Ser	V	Tyr	Arg	0.22	51
huMAb4D5-7	Ala	Thr	Ala	Ser	Tyr	E	Arg	0.62	53
huMAb4D5-8	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.10	54
muMAb4D5	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.30	37

*Human and murine residues are shown in one letter and three letter amino acid code respectively.
†K_d values for the p185^{HER2} ECD were determined using the method of Friguet et al. (43) and the standard error of each estimate is ≦ ± 10%.
‡Proliferation of SK-BR-3 cells incubated for 96 hr with MAb4D5 variants shown as a percentage of the untreated control as described (Hudziak, R. M. et al., Molec. Cell. Biol. 9: 1165–1172 (1989)). Data represent the maximal anti-proliferative effect for each variant (see FIG. 3A) calculated as the mean of triplicate determinations at a MAb4D5 concentration of 8 μg/ml. Data are all taken from the same experiment with an estimated standard error of ≦ ± 15%.

TABLE 4

Selectivity of antibody dependent tumor cell cytotoxicity mediated by huMAb4D5-8

Effector:Target ratio†	WI-38* muMAb4D5	WI-38* huMAb4D5-8	SK-BR-3 muMAb4D5	SK-BR-3 huMAb4D5-8
A.‡				
25:1	<1.0	9.3	7.5	40.6
12.5:1	<1.0	11.1	4.7	36.8
6.25:1	<1.0	8.9	0.9	35.2
3.13:1	<1.0	8.5	4.6	19.6
B.				
25:1	<1.0	3.1	6.1	33.4
12.5:1	<1.0	1.7	5.5	26.2
6.25:1	1.3	2.2	2.0	21.0
3.13:1	<1.0	0.8	2.4	13.4

*Sensitivity to ADCC of two human cell lines (WI-38, normal lung epithelium; and SK-BR-3, human breast tumor cell line) are compared. WI-38 expresses a low level of p185^{HER2} (0.6 pg per μg cell protein) and SK-BR-3 expresses a high level of p185^{HER2} (64 pg p185^{HER2} per μg cell protein), as determined by ELISA (Fendly et al., J. Biol. Resp. Mod. 9:449–455 (1990)).
†ADCC assays were carried out as described in Brüggemann et al., J. Exp. Med. 166:1351–1361 (1987). Effector to target ratios were of IL-2 activated human peripheral blood lymphocytes to either WI-38 fibroblasts or SK-BR-3 tumor cells in 96-well microtiter plates for 4 hours at 37° C. Values given represent percent specific cell lysis as determined by ^{51}Cr release. Estimated standard error in these quadruplicate determinations was ≦±10%.
‡Monoclonal antibody concentrations used were 0.1 μg/ml (A) and 0.1 μg/ml (B).

Example 2
Schematic Method for Humanizing an Antibody Sequence

This example illustrates one stepwise elaboration of the methods for creating a humanized sequence described

above. It will be understood that not all of these steps are essential to the claimed invention, and that steps may be taken in different order.

1. ascertain a consensus human variable domain amino acid sequence and prepare from it a consensus structural model.
2. prepare model of import (the non-human domain to be humanized) variable domain sequences and note structural differences with respect to consensus human model.
3. identify CDR sequences in human and in import, both by using Kabat (supra, 1987) and crystal structure criteria. If there is any difference in CDR identity from the different criteria, use of crystal structure definition of the CDR, but retain the Kabat residues as important framework residues to import.
4. substitute import CDR sequences for human CDR sequences to obtain initial "humanized" sequence.
5. compare import non-CDR variable domain sequence to the humanized sequence and note divergences.
6. Proceed through the following analysis for each amino acid residue where the import diverges from the humanized.
   a. If the humanized residue represents a residue which is generally highly conserved across all species, use the residue in the humanized sequence. If the residue is not conserved across all species, proceed with the analysis described in 6b.
   b. If the residue is not generally conserved across all species, ask if the residue is generally conserved in humans.
      i. If the residue is generally conserved in humans but the import residue differs, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs by considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, leave the humanized residue unchanged.
      ii. If the residue is also not generally conserved in humans, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs be considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, proceed to the next step.
         a) Examine the structural models of the import and human sequences and determine if the residue is exposed on the surface of the domain or is buried within. If the residue is exposed, use the residue in the humanized sequence. If the residue is buried, proceed to the next step.
            (i) Examine the structural models of the import and human sequences and determine if the residue is likely to affect the V_L–V_H interface. Residues involved with the interface include: 34L, 36L, 38L, 43L, 33L, 36L, 85L, 87L, 89L, 91L, 96L, 98L, 35H, 37H, 39H, 43H, 45H, 47H, 60H, 91H, 93H, 95H, 100H, and 103H. If no effect is likely, use the residue in the humanized sequence. If some affect is likely, substitute the import residue.
7. Search the import sequence, the consensus sequence and the humanized sequence for glycosylation sites outside the CDRs, and determine if this glycosylation site is likely to have any affect on antigen binding and/or biological activity. If no effect is likely, use the human sequence at that site; if some affect is likely, eliminate the glycosylation site or use the import sequence at that site.
8. After completing the above analysis, determine the planned humanized sequence and prepare and test a sample. If the sample does not bind well to the target antigen, examine the particular residues listed below, regardless of the question of residue identity between the import and humanized residues.
   a. Examine particular peripheral (non-CDR) variable domain residues that may, due to their position, possibly interact directly with a macromolecular antigen, including the following residues (where the * indicates residues which have been found to interact with antigen based on crystal structures):
      i. Variable light domain: 36, 46, 49*, 63–70
      ii. Variable heavy domain: 2, 47*, 68, 70, 73–76.
   b. Examine particular variable domain residues which could interact with, or otherwise affect, the conformation of variable domain CDRs, including the following (not including CDR residues themselves, since it is assumed that, because the CDRs interact with one another, any residue in one CDR could potentially affect the conformation of another CDR residue) (L=LIGHT, H=HEAVY, residues appearing in bold are indicated to be structurally important according the Chothia et al., Nature 342:877 (1989), and residues appearing in italic were altered during humanization by Queen et al. (PDL), Proc. Natl. Acad. Sci. USA 86:10029 (1989) and Proc. Natl. Acad. Sci. USA 88:2869 (1991).):
      i. Variable light domain:
         a) CDR-1 (residues 24L–34L): 2L, 4L, 66L-69L, 71L
         b) CDR-2 (residues 50L–56L): 35L, 46L, 47L, 48L, 49L, 58L, 62L, 64L–66L, 71L, 73L
         c) CDR-3 (residues 89L–97L): 2L, 4L, 36L, 98L, 37H, 45H, 47H, 58H, 60H
      ii. Variable heavy domain:
         a) CDR-1 (residues 26H–35H): 2H, 4H, 24H, 36H, 71H, 73H, 76H, 78H, 92H, 94H
         b) CDR-2 (residues 50H–55H): 49H, 69H, 69H, 71H, 73H, 78H
         c) CDR-3 (residues 95H–102H): examine all residues as possible interaction partners with this loop, because this loop varies in size and conformation much more than the other CDRs.
9. If after step 8 the humanized variable domain still is lacking in desired binding, repeat step 8. In addition, re-investigate any buried residues which might affect the V_L–V_H interface (but which would not directly affect CDR conformation). Additionally, evaluate the accessibility of non-CDR residues to solvent.

Example 3

Engineering a Humanized Bisnecific F(ab')$_2$ Fragment

This example demonstrates the construction of a humanized bispecific antibody (BsF(ab')$_2$v1 by separate *E. coli* expression of each Fab' arm followed by directed chemical coupling in vitro. BsF(ab')$_2$v1 (anti-CD3/anti-p185^{HER2}) was demonstrated to retarget the cytotoxic activity of human

CD3+CTL in vitro against the human breast tumor cell line, SK-BR-3, which overexpresses the p185^{HER2} product of the protooncogene HER2. This example demonstrates the minimalistic humanization strategy of installing as few murine residues as possible into a human antibody in order to recruit antigen-binding affinity and biological properties comparable to that of the murine parent antibody. This strategy proved very successful for the anti-p185^{HER2}arm of BsF(ab')$_2$v1. In contrast BsF(ab')$_2$ v1 binds to T cells via its anti-CD3 arm much less efficiently than does the chimeric BsF(ab')$_2$ which contains the variable domains of the murine parent anti-CD3 antibody. Here we have constructed additional BsF(ab')$_2$ fragments containing variant anti-CD3 arms with selected murine residues restored in an attempt to improve antibody binding to T cells. One such variant, Ss F(ab')$_2$v9, was created by replacing six residues in the second hypervariable loop of the anti-CD3 heavy chain variable domain of BsF(ab')$_2$v1 with their counterparts from the murine parent anti-CD3 antibody. BsF(ab')$_2$v9 binds to T cells (Jurkat) much more efficiently than does BsF(ab')$_2$v1 and almost as efficiently as the chimeric BsF(ab')$_2$. This improvement in the efficiency of T cell binding of the humanized BsF(ab')$_2$ is an important step in its development as a potential therapeutic agent for the treatment of p185^{HER2}-overexpressing cancers.

Bispecific antibodies (BsAbs) with specificities for tumor-associated antigens and surface markers on immune effector cells have proved effective for retargeting effector cells to kill tumor targets both in vitro and in vivo (reviewed by Fanger, M. W. et al., *Immunol. Today* 10: 92–99 (1989); Fanger, M. W. et al., *Immunol. Today* 12: 51–54 (1991); and Nelson, H., *Cancer Cells* 3: 163–172 (1991)). BsF(ab')$_2$ fragments have often been used in preference to intact BsAbs in retargeted cellular cytotoxicity to avoid the risk of killing innocent bystander cells binding to the Fc region of the antibody. An additional advantage of BsF(ab')$_2$ over intact BsAbs is that they are generally much simpler to prepare free of contaminating monospecific molecules (reviewed by Songsivilai, S. and Lachmann, P. J., *Clin. Exp. Immunol.* 79: 315–321 (1990) and Nolan, O. and O'Kennedy, R., *Biochim. Biophys. Acta* 1040: 1–11 (1990)).

BsF(ab')$_2$ fragments are traditionally constructed by directed chemical coupling of Fab' fragments obtained by limited proteolysis plus mild reduction of the parent rodent monoclonal Ab (Brennan, M. et al., *Science* 229, 81–83 (1985) and Glennie, M. J. et al., *J. Immunol.* 139: 2367–2375 (1987)). One such BsF(ab')$_2$ fragment (anti-glioma associated antigen/anti-CD3) was found to have clinical efficacy in glioma patients (Nitta, T. et al., *Lancet* 335: 368–371 (1990) and another BsF(ab')$_2$ (anti-indium chelate/anti-carcinoembryonic antigen) allowed clinical imaging of colorectal carcinoma (Stickney, D. R. et al., *Antibody, Immunoconj. Radiopharm.* 2: 1–13 (1989)). Future SsF(ab')$_2$ destined for clinical applications are likely to be constructed from antibodies which are either human or at least "humanized" (Riechmann, L. et al., *Nature* 332: 323–327 (1988) to reduce their immunogenicity (Hale, G. et al., *Lancet i:* 1394–1399 (1988)).

Recently a facile route to a fully humanized BsF(ab')$_2$ fragment designed for tumor immunotherapy has been demonstrated (Shalaby, M. R. et al., *J. Exp. Med.* 175: 217–225 (1992)). This approach involves separate *E. coli* expression of each Fab' arm followed by traditional directed chemical coupling in vitro to form the BsF(ab')$_2$. One arm of the BsF(ab')$_2$ was a humanized version (Carter, P. et al., *Proc. Natl. Aced. Sci. USA* (1992a) and Carter, P., et al., *Bio/Technology* 10: 163–167 (1992b)) of the murine monoclonal Ab 4D5 which is directed against the p185^{HER2} product of the protooncogene HER2 (c-erbB-2) (Fendly, B. M. et al. *Cancer Res.* 50: 1550–1558 (1989)). The humanization of the antibody 4D5 is shown in Example 1 of this application. The second arm was a minimalistically humanized anti-CD3 antibody (Shalaby et al. supra) which was created by installing the CDR loops from the variable domains of the murine parent monoclonal Ab UCHT1 (Beverley, P. C. L. and Callard, R. E., *Eur. J. Immunol.* 11: 329–334 (1981)) into the humanized anti-p185^{HER2} antibody. The BsF(ab')$_2$ fragment containing the most potent humanized anti-CD3 variant (v1) was demonstrated by flow cytometry to bind specifically to a tumor target overexpressing p185^{HER2} and to human peripheral blood mononuclear cells carrying CD3. In addition, BsF(ab')$_2$v1 enhanced the cytotoxic effects of activated human CTL 4-fold against SK-SR-3 tumor cells overexpressing p185^{HER2}. The example descries efforts to improve the antigen binding affinity of the humanized anti-CD3 arm by the judicious recruitment of a small number of additional murine residues into the minimalistically humanized anti-CD3 variable domains.

Materials and Methods

Construction of Mutations in the Anti-CD3 Variable Region Genes

The construction of genes encoding humanized anti-CD3 variant 1 (v1) variable light (V_1) and heavy (V_H) chain domains in phagemid pUC119 has been described (Shalaby et al. supra). Additional anti-CD3 variants were generated using an efficient site-directed mutagenesis method (Carter, P., *Mutagenesis: a practical approach,* (M. J. McPherson, Ed.), Chapter 1, IRL Press, Oxford, UK (1991)) using mismatched oligonucleotides which either install or remove unique restriction sites. Oligonucleotides used are listed below using lowercase to indicate the targeted mutations. Corresponding coding changes are denoted by the starting amino acid in one letter code followed by the residue numbered according to Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest,* 5th edition, National Institutes of Health, Bethesda, Md., USA (1991), then the replacement amino acid and finally the identity of the anti-CD3 variant:

HX11, 5' GTAGATAAATCCtctAACACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 11) V_HK75S, v6;

HX12, 5' GTAGATAAATCCAAAtctACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 12) V_H N76S, v7;

HX13, 5' GTAGATAAATCCtcttctACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 13) V_H K75S:N76S, v8;

X14, 5' CTTATAAAGGTGTTtCcACCTATaaCcAgAaatTCAAGGatCGTTTCACgATAtcCGTAGATAAATCC 3' (SEO.ID.NO. 14) V_H T57S:A60N:D61Q:S62K:V63F:G65D, v9;

LX6, 5' CTATACCTCCCGTCTgcatTCTGGAGTCCC 3' (SEQ.ID. NO. 15) V_L E55H, v11.

Oliconucleotides HX11, HX12 and HX13 each remove a site for BspMI, whereas LX6 removes a site for XhoI and HX14 installs a site for EcoRV (bold). Anti-CD3 variant v10 was constructed from v9 by site-directed mutagenesis using oligonucleotide HX13. Mutants were verified by dideoxynucleotide sequencing (Sanger, F. et al., *Proc. Natl. Acad, Sci. USA* 74: 5463–5467 (1977)).

E. coli Expression of Fab' Fragments

The expression plasmid, pAK19, for the co-secretion of light chain and heavy chain Fd' fragment of the most preferred humanized anti-p185^{HER2} variant, HuMAb4D5-8, is described in Carter et al., 1992b, supre. Briefly, the Fab' expression unit is bicistronic with both chains under the

transcriptional control of the ohoA promoter. Genes encoding humanized V_L and V_H domains are precisely fused on their 5' side to a gene segment encoding the heat-stable enterotoxin II signal sequence and on their 3' side to human k_1 C_L and IgG1C_H1 constant domain genes, respectively. The C_H1gene is immediately followed by a sequence encoding the hinge sequence CysAlaAla and followed by a bacteriophage λ t_o transcriptional terminator. Fab' expression plasmids for chimeric and humanized anti-CD3 variants (v1 to v4, Shalaby et al.,supra; v6 to v12, this study) were created from pAK19 by precisely replacing anti-p185^{HER2} V_L and V_H gene segments with those encoding murine and corresponding humanized variants of the anti-CD3 antibody, respectively, by sub-cloning and site-directed mutagenesis. The Fab' expression plasmid for the most potent humanized anti-CD3 variant identified in this study (v9) is designated pAK22. The anti-p185^{HER2} Fab' fragment was secreted from *E. coli* K12 strain 25F2 containing plasmid pAK19 grown for 32 to 40 hr at 37° C. in an aerated 10 liter fermentor. The final cell density was 120–150 OD_{550} and the titer of soluble and functional anti-p185^{HER2} Fab' was 1–2 g/liter as judged by antigen binding ELISA (Carter et al., 1992b, suora). Anti-CD3 Fab' variants were secreted from *E. coli* containing corresponding expression plasmids using very similar fermentation protocols. The highest expression titers of chimeric and humanized anti-CD3 variants were 200 mgaliter and 700 mgaliter, respectively, as judged by total immunoglobulin ELISA.

Construction of BsF(ab')$_2$ Fragments

Fab' fragments were directly recovered from *E. coli* fermentation pastes in the free thiol form (Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b supra). Thioether linked BsF(ab')$_2$ fragments (anti-p185^{HER2}/anti-CD3) were constructed by the procedure of Glennie et al. supra with the following modifications. Anti-p185^{HER2} Fab'-SH in 100 mM Tris acetate, 5 mM EDTA (pH 5.0) was reacted with 0.1 vol of 40 mM N,N'-1,2-phenylenedimalemide (o-PDM) in dimethyl formamide for ~1.5 hr at 20° C. Excess o-PDM was removed by protein G purification of the Fab' maleimide derivative (Fab'-mal) followed by buffer exchange into 20 mM sodium acetate, 5 mM EDTA (pH 5.3) (coupling buffer) using centriprep-30 concentrators (Amicon). The total concentration of Fab' variants was estimated from the measured absorbance at 280 nm (HuMAb4D5-8 Fab' $e^{0.1}$%=1.56, Carter et al., 1992b, supra). The free thiol content of Fab' preparations was estimated by reaction with 5,5' -dithiobis (2-nitrobenzoic acid) as described by Creighton, T. E., *Protein structure: a practical approach,* (T. E. Creighton, Ed.), Chapter 7, IRL Press, Oxford, UK (1990). Equimolar amounts of anti-p185^{HER2} Fab'-mal (assuming quantitative reaction of Fab'-SH with o-PDM) and each anti-CD3 Fab'-SH variant were coupled together at a combined concentration of 1 to 2.5 mg/ml in the coupling buffer for 14 to 48 hr at 4° C. The coupling reaction was adjusted to 4 mM cysteine at pH 7.0 and incubated for 15 min at 20 ° C. to reduce any unwanted disulfide-linked F(ab')$_2$ formed. These reduction conditions are sufficient to reduce inter-heavy chain disulfide bonds with virtually no reduction of the disulfide between light and heavy chains. Any free thiols generated were then blocked with 50 mM iodoacetamide. BsF(ab')$_2$ was isolated from the coupling reaction by S100-HR (Pharmacia) size exclusion chromatography (2.5 cm×100 cm) in the presence of PBS. The BsF(ab')$_2$ samples were passed through a 0.2 mm filter flash frozen in liquid nitrogen and stored at −70° C.

Flow Cytometric Analysis of F(ab')$_2$Binding to Jurkat Cells

The Jurkat human acute T cell leukemia cell line was purchased from the American Type Culture Collection (Manassas Va.) (ATCC TIB 152) and grown as. recommended by the ATCC. Aliquots of 10^6 Jurkat cells were incubated with appropriate concentrations of BsF(ab')$_2$ (anti-p185^{HER2}/anti-CD3 variant) or control mono-specific anti-p185^{HER2} F(ab')$_2$ in PBS plus 0.1% (w/v) bovine serum albumin and 10 mM sodium azide for 45 min at 4° C. The cells were washed and then incubated with fluorescein-conjugated goat anti-human F(ab')$_2$ (Organon Teknika, West Chester, Pa.) for 45 min at 4° C. Cells were washed and analyzed on a FACScan® (Becton Dickinson and Co., Mountain View, Calif.). Cells (8×10^3) were acquired by list mode and gated by forward light scatter versus side light scatter excluding dead cells and debris.

Results

Design of Humanized anti-CD3 Variants

The most potent humanized anti-CD3 variant previously identified, v1, differs from the murine parent antibody, UCHT1 at 19 out of 107 amino acid residues within V_L and at 37 out of 122 positions within V_H (Shalaby et al.,supra) 1992). Here we recruited back additional murine residues into anti-CD3 v1 in an attempt to improve the binding affinity for CD3. The strategy chosen was a compromise between minimizing both the number of additional murine residues recruited and the number of anti-CD3 variants to be analyzed. We focused our attentions on a few CDR residues which were originally kept as human sequences in our minimalistic humanization regime. Thus human residues in V_H CDR2 of anti-CD3 v1 were replaced en bloc with their murine counterparts to give anti-CD3 v9: T57S:A60N:D61Q:S62K:V63F:G65D (SEQ ID NO:20). Similarly, the human residue E55 in V_L CDR2 of anti-CD3 v_1 was replaced with histidine from the murine anti-CD3 antibody to generate anti-CD3 v11. In addition, V_H framework region (FR) residues 75 and 76 in anti-CD3 v1 were also replaced with their murine counterparts to create anti-CD3 v8: K75S:N76S. V_H residues 75 and 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might influence antigen binding. Additional variants created by combining mutations at these three sites are described below.

Preparation of BsF(ab')$_2$ Fragments

Soluble and functional anti-p185^{HER2} and anti-CD3 Fab' fragments were recovered directly from corresponding *E. coli* fermentation pastes with the single hinge cysteine predominantly in the free thiol form (75–100% Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b, supra). Thioether-linked BsF(ab')$_2$ fragments were then constructed by directed coupling using o-PDM as described by Glennie et al., supra. One arm was always the most potent humanized anti-p185^{HER2} variant, HuMAb4D5-8 (Carter et al., 1992a, supra) and the other either a chimeric or humanized variant of the anti-CD3 antibody. Anti-p185^{HER2} Fab'-SH was reacted with o-PDM to form the maleimide derivative (Fab'-mal) and then coupled to the Fab'-SH for each anti-CD3 variant. F(ab')$_2$ was then purified away from unreacted Fab' by size exclusion chromatography as shown for a representative preparation (BsF(ab')$_2$ v8) in data not shown. The F(ab')$_2$ fragment represents ~54% of the total amount of antibody fragments (by mass) as judged by integration of the chromatograph peaks.

SDS-PAGE analysis of this BsF(ab')$_2$v8 preparation under non-reducing conditions gave one major band with the expected mobility (M, ~96 kD) as well as several very minor

bands (data not shown). Amino-terminal sequence analysis of the major band after electroblotting on to polyvinylidene difluoride 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might membrane Matsudaira, P., *J. Biol. Chem.* 262: 10035–10038 (1987) gave the expected mixed sequence from a stoichiometric 1:1 mixture of light and heavy chains (V_L/V_H: D/E, I/V, Q/D, M/L, T/V, D/E, S/S) expected for BsF(ab')$_2$. The amino terminal region of both light chains are identical as are both heavy chains and correspond to consensus human FR sequences. We have previously demonstrated that F(ab')$_2$ constructed by directed chemical coupling carry both anti-p185^{HER2} and anti-CD3 antigen specificities (Shalaby et al., supra). The level of contamination of the BsF(ab')$_2$ with monospecific F(ab')$_2$ iS likely to be very low since mock coupling reactions with either anti-p185^{HER2} w Fab'-mal or anti-CD3 Fab'-SH alone did not yield detectable quantities of F(ab')$_2$. Furthermore the coupling reaction was subjected to a mild reduction step followed by alkylation to remove trace amounts of disulfide-linked F(ab')$_2$ that might be present. SDS-PAGE of the purified F(ab')$_2$ under reducing conditions gave two major bands with electrophoretic mobility and amino terminal sequence anticipated for free light chain and thioether-linked heavy chain dimers.

Scanning LASER densitometry of a o-PDM coupled F(ab')$_2$ preparation suggest that the minor species together represent ~10% of the protein. These minor contaminants were characterized by amino terminal sequence analysis and were tentatively identified on the basis of stoichiometry of light and heavy chain sequences and their electrophoretic mobility (data not shown). These data are consistent with the minor contaminants including imperfect F(ab')$_2$ in which the disulfide bond between light and heavy chains is missing in one or both arms, trace amounts of Fab' and heavy chain thioether-linked to light chain.

Binding of BsF(ab')$_2$ to Jurkat Cells

Binding of BsF(ab')$_2$ containing different anti-CD3 variants to Jurkat cells (human acute T cell leukemia) was investigated by flow cytometry (data not shown). BsF(ab')$_2$v9 binds much more efficiently to Jurkat cells than does our starting molecule, BsF(ab')$_2$vl, and almost as efficiently as the chimeric BsF(ab')$_2$. Installation of additional murine residues into anti-CD3 v9 to create v10 (V_HK75S:N76S) and v12 (V_HK75S:N76S plus V_L E55H) did not further improve binding of corresponding BsF(ab')$_2$to Jurkat cells. Nor did recruitment of these murine residues into anti-CD3 v1 improve Jurkat binding: V_HK75S (v6), V_HN76S (v7), V_HK75S:N76S (V8), V_LE55H (v11) (not shown). BsF(ab')$_2$v9 was chosen for future study since it is amongst the most efficient variants in binding to Jurkat cells and contains fewest murine residues in the humanized anti-CD3 arm. A monospecific anti-p185^{HEE2} F(ab')$_2$ did not show significant binding to Jurkat cells consistent with the interaction being mediated through the anti-CD3 arm.

Discussion

A minimalistic strategy was chosen to humanize the anti-p185^{HER2} (Carter et al., 1992a, supra) and anti-CD3 arms (Shalaby et al., supra) of the BsF(ab')$_2$ in this study in an attempt to minimize the potential immunogenicity of the resulting humanized antibody in the clinic. Thus we tried to install the minimum number of murine CDR and FR residues into the context of consensus human variable domain sequences as required to recruit antigen-binding affinity and biological properties comparable to the murine parent antibody. Molecular modeling was used firstly to predict the murine FR residues which might be important to antigen binding and secondly to predict the murine CDR residues that might not be required. A small number of humanized variants were then constructed to test these predictions.

Our humanization strategy was very successful for the anti-p185^{HER2} antibody where one out of eight humanized variants (HuMAb4D5-8, IgG1) was identified that bound the p185^{HER2} antigen ~3-fold more tightly than the parent murine antibody (Carter et al., 1992a, supra). HuMAb4D5-8 contains a total of five murine FR residues and nine murine CDR residues, including V_H CDR2 residues 60–65, were discarded in favor of human counterparts. In contrast, BsF(ab')$_2$v1 containing the most potent humanized anti-CD3 variant out of four originally constructed (Shalaby et al., supra) binds J6 cells with an affinity (K_d) of 140 nM which is ~70-fold weaker than that of the corresponding chimeric BsF(ab')$_2$.

Here we have restored T cell binding of the humanized anti-CD3 close to that of the chimeric variant by replacing six human residues in V_H CDR2 with their murine counterparts: T57S:A60N:D61Q:S62K:V63F:G65D (anti-CD3 v9, FIG. **5**). It appears more likely that these murine residues enhance antigen binding indirectly by influencing the conformation of residues in the N-terminal part of V_H CDR2 rather than by directly contacting antigen. Firstly, only N-terminal residues in V_H CDR2 (50–58) have been found to contact antigen in one or more of eight crystallographic structures of antibody/antigen complexes (Kabat et al., supra; and Mian, I. S. et al., *J. Mol. Biol* 217: 133–151 (1991), FIG. **5**). Secondly, molecular modeling suggests that residues in the C-terminal part of V_H CDR2 are at least partially buried (FIG. **5**). BsF(ab')$_2$v9 binds to SK-BR-3 breast tumor cells with equal efficiency to BsF(ab')$_2$v1 and chimeric BsF(ab')$_2$ as anticipated since the anti-p185^{HER2} arm is identical in all of these molecules (Shalaby et al., supra, not shown).

Our novel approach to the construction of BsF(ab')$_2$ fragments exploits an *E. coli* expression system which secretes humanized Fab' fragments at gram per liter titers and permits their direct recovery as Fab'-SH (Carter et al., 1992b, supra). Traditional directed chemical coupling of Fab'-SH fragments is then used to form BsF(ab')$_2$ in vitro (Brennan et al.,supra; and Glennie et al., supra). This route to Fab'-SH obviates problems which are inherent in their generation from intact antibodies: differences in susceptibility to proteolysis and nonspecific cleavage resulting in heterogeneity, low yield as well as partial reduction that is not completely selective for the hinge disulfide bonds. The strategy of using *E. coli*-derived Fab'-SH containing a single hinge cysteine abolishes some sources of heterogeneity in BsF(ab')$_2$ preparation such as intra-hinge disulfide formation and contamination with intact parent antibody whilst greatly diminishes others, eg. formation of F(ab')$_3$ fragments.

BsF(ab')2 fragments constructed here were thioether-linked as originally described by Glennie et al., supra with future in vivo testing of these molecules in mind. Thioether bonds, unlike disulfide bonds, are not susceptible to cleavage by trace amounts of thiol, which led to the proposal that thioether-linked F(ab')$_2$ may be more stable than disulfide-linked F(ab')$_2$ in vivo (Glennie et al., supra). This hypothesis is supported by our preliminary pharmacokinetic experiments in normal mice which suggest that thioether-linked BsF(ab')$_2$ v1 has a 3-fold longer plasma residence time than BsF(ab')$_2$ v1 linked by a single disulfide bond. Disulfide and thioether-linked chimeric BsF(ab')$_2$ were found to be indistinguishable in their efficiency of cell binding and in their retargeting of CTL cytotoxicity, which suggests that o-PDM directed coupling does not compromise binding of the

BsF(ab')$_2$ to either antigen (not shown). Nevertheless the nature of the linkage appears not to be critical since a disulfide-linked BsF(ab')$_2$ (murine anti-p185^{HER2}/murine anti-CD3) was recently shown by others (Nishimura et al., Int. *J. Cancer* 50: 800–804 (1992) to have potent anti-tumor activity in nude mice. Our previous study (Shalaby et al., supra) together with this one and that of Nishimura, T. et al., supra improve the potential for using BsF(ab')$_2$ in targeted immunotherapy of p185^{HER2}-overexpressing cancers in humans.

Example 4

Humanization of an anti-CD18 Antibody

A murine antibody directed against the leukocyte adhesion receptor β-chain (known as the H52 antibody) was humanized following the methods described above. FIGS. **6**A and **6**B provide amino acid sequence comparisons for the murine and humanized antibody light chains and heavy chains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 1 00                 1 05

Ile Lys Arg Thr
            109
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                65                  70                  75
```

-continued

```
Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                 95                 1 00                 1 05

Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                  120
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Ser
                 20                  25                  30

Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

Tyr Asn Ser Leu Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 1 00                 1 05

Ile Lys Arg Thr
            109
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                 20                  25                  30

Asp Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Val Ile Ser Glu Asn Gly Ser Asp Thr Tyr Tyr
                 50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser
                 65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asp Arg Gly Gly Ala Val Ser
                 95                 1 00                 1 05

Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                  120
```

-continued

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val
1 5 10 15

Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Asn
20 25 30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly His Ser Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Phe Arg Tyr Thr Gly Val Pro Asp
50 55 60

Arg Phe Thr Gly Asn Arg Ser Gly Thr Asp Phe Thr Phe Thr Ile
65 70 75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
80 85 90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu
95 1 00 1 05

Ile Lys Arg Ala
109

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Ala Ser Leu Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
20 25 30

Asp Thr Tyr Ile His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu
35 40 45

Glu Trp Ile Gly Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
50 55 60

Asp Pro Lys Phe Gln Asp Lys Ala Thr Ile Thr Ala Asp Thr Ser
65 70 75

Ser Asn Thr Ala Tyr Leu Gln Val Ser Arg Leu Thr Ser Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
95 1 00 1 05

Ala Met Asp Tyr Trp Gly Gln Gly Ala Ser Val Thr Val Ser Ser
110 115 120

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCCGATATCC AGCTGACCCA GTCTCCA 27

-continued

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTTGATCTC CAGCTTGGTA CCHSCDCCGA A 31

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGGTSMARCT GCAGSAGTCW GG 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAG 34

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTAGATAAAT CCAAATCTAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTAGATAAAT CCTCTTCTAC AGCCTATCTG CAAATG 36

-continued

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTATAAAGG TGTTTCCACC TATAACCAGA AATTCAAGGA TCGTTTCACG 50

ATATCCGTAG ATAAATCC 68

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTATACCTCC CGTCTGCATT CTGGAGTCCC 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
1 5 10 15

Gly Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Arg
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys
35 40 45

Leu Leu Ile Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser
50 55 60

Lys Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
65 70 75

Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
80 85 90

Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly Gly Thr Lys Leu Glu
95 1 00 1 05

Ile Lys
107

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Arg
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys

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                35                  40                  45

 Leu Leu Ile Tyr Tyr Thr Ser Arg Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
                65                  70                  75

 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

 Gly Asn Thr Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 1 00                1 05

 Ile Lys
     107


(2) INFORMATION FOR SEQ ID NO:18:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 107 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
   1               5                  10                  15

 Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
                20                  25                  30

 Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

 Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

 Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 1 00                1 05

 Ile Lys
     107


(2) INFORMATION FOR SEQ ID NO:19:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 122 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

 Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
   1               5                  10                  15

 Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr
                20                  25                  30

 Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu
                35                  40                  45

 Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
                50                  55                  60

 Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys Ser
                65                  70                  75

 Ser Ser Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp
                80                  85                  90
```

-continued

Ser Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
95 1 00 1 05

Asp Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val
110 115 120

Ser Ser
122

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
20 25 30

Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
50 55 60

Asn Gln Lys Phe Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
65 70 75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
95 1 00 1 05

Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
110 115 120

Ser Ser
122

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
20 25 30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
50 55 60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
95 1 00 1 05

Ser Gly Leu Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
110 115 120

-continued

Ser Ser
122

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr
20 25 30

Glu Tyr Thr Met His Trp Met Lys Gln Ser His Gly Lys Ser Leu
35 40 45

Glu Trp Ile Gly Gly Phe Asn Pro Lys Asn Gly Gly Ser Ser His
50 55 60

Asn Gln Arg Phe Met Asp Lys Ala Thr Leu Ala Val Asp Lys Ser
65 70 75

Thr Ser Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu Asp
80 85 90

Ser Gly Ile Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
95 1 00 1 05

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val
110 115 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
125 130 135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
140 145 150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
155 160 165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
170 175 180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
185 190 195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
200 205 210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
215 220 225

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
230 235 240

Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
245 250 255

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
260 265 270

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
275 280 285

Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
290 295 300

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
305 310 315

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
320 325 330

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Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
                335                 340                 345

Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
                350                 355                 360

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
                365                 370                 375

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
                380                 385                 390

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
                395                 400                 405

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
                410                 415                 420

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
                425                 430                 435

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
                440                 445                 450

Ser Pro Gly Lys
            454


(2) INFORMATION FOR SEQ ID NO:23:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 469 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
  1               5                  10                  15

Gly Val His Ser Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu
                20                  25                  30

Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly
                35                  40                  45

Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro
                50                  55                  60

Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly
                65                  70                  75

Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser
                80                  85                  90

Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu
                95                 1 00                1 05

Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly
                110                 115                 120

Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln
                125                 130                 135

Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
                140                 145                 150

Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
                155                 160                 165

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
                170                 175                 180

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr
                185                 190                 195

Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser
                200                 205                 210
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-continued

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Val Val Thr Val Thr Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr
                215                 220                 225

Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr
                230                 235                 240

Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
                245                 250                 255

Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
                260                 265                 270

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
                275                 280                 285

Val Asp Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr
                290                 295                 300

Val Asp Gly Met Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
                305                 310                 315

Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu Thr Val
                320                 325                 330

Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
                335                 340                 345

Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
                350                 355                 360

Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
                365                 370                 375

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
                380                 385                 390

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
                395                 400                 405

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu
                410                 415                 420

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
                425                 430                 435

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
                440                 445                 450

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
                455                 460                 465

Ser Pro Gly Lys
            469


(2) INFORMATION FOR SEQ ID NO:24:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 214 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Asp Val Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Asn Cys Arg Ala Ser Gln Asp Ile Asn
                20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asn Gly Thr Val Lys
                35                  40                  45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
                65                  70                  75
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Ser Asn Leu Asp Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
                 80                  85                  90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gly Gly Thr Lys Val Glu
                 95                 1 00                 1 05

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys
            214
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

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Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
  1               5                  10                  15

Gly Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu
                 20                  25                  30

Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
                 35                  40                  45

Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly
                 50                  55                  60

Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser
                 65                  70                  75

Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr
                 80                  85                  90

Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr
                 95                 1 00                 1 05

Tyr Cys Gln Gln Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly
                110                 115                 120

Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe
                125                 130                 135

Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser
                140                 145                 150

Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val
                155                 160                 165

Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
                170                 175                 180

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                185                 190                 195
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-continued

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Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val
                200                 205                 210

Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr
                215                 220                 225

Lys Ser Phe Asn Arg Gly Glu Cys
                230          233


(2) INFORMATION FOR SEQ ID NO:26:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 122 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
                20                  25                  30

Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Thr Thr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
                65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                95                 1 00                1 05

Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
               110                 115                 120

Ser Ser
    122
```

We claim:

1. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, and 92H, utilizing the numbering system set forth in Kabat.

2. The humanized variable domain of claim **1** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

3. The humanized variable domain of claim **1** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

4. The humanized variable domain of claim **1** wherein the human antibody variable domain is a consensus human variable domain.

5. The humanized variable domain of claim **1** wherein the residue at site 4L has been substituted.

6. The humanized variable domain of claim **1** wherein the residue at site 38L has been substituted.

7. The humanized variable domain of claim **1** wherein the residue at site 43L has been substituted.

8. The humanized variable domain of claim **1** wherein the residue at site 44L has been substituted.

9. The humanized variable domain of claim **1** wherein the residue at site 58L has been substituted.

10. The humanized variable domain of claim **1** wherein the residue at site 62L has been substituted.

11. The humanized variable domain of claim **1** wherein the residue at site 65L has been substituted.

12. The humanized variable domain of claim **1** wherein the residue at site 66L has been substituted.

13. The humanized variable domain of claim **1** wherein the residue at site 67L has been substituted.

14. The humanized variable domain of claim **1** wherein the residue at site 68L has been substituted.

15. The humanized variable domain of claim **1** wherein the residue at site 69L has been substituted.

16. The humanized variable domain of claim **1** wherein the residue at site 73L has been substituted.

17. The humanized variable domain of claim **1** wherein the residue at site 85L has been substituted.

18. The humanized variable domain of claim **1** wherein the residue at site 98L has been substituted.

19. The humanized variable domain of claim **1** wherein the residue at site 2H has been substituted.

20. The humanized variable domain of claim **1** wherein the residue at site 4H has been substituted.

21. The humanized variable domain of claim **1** wherein the residue at site 36H has been substituted.

22. The humanized variable domain of claim **1** wherein the residue at site 39H has been substituted.

23. The humanized variable domain of claim **1** wherein the residue at site 43H has been substituted.

24. The humanized variable domain of claim **1** wherein the residue at site 45H has been substituted.

25. The humanized variable domain of claim **1** wherein the residue at site 69H has been substituted.

26. The humanized variable domain of claim **1** wherein the residue at site 70H has been substituted.

27. The humanized variable domain of claim **1** wherein the residue at site 74H has been substituted.

28. The humanized variable domain of claim **1** wherein the residue at site 92H has been substituted.

29. An antibody comprising the humanized variable domain of claim **1**.

30. An antibody which binds $p185^{HER2}$ and comprises a humanized antibody variable domain, wherein the humanized antibody variable domain comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind $p185^{HER2}$ incorporated into a human antibody variable domain, and further comprises a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

31. The antibody of claim **30** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

32. The antibody of claim **30** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

33. The antibody of claim **30** wherein the human antibody variable domain is a consensus human variable domain.

34. The antibody of claim **30** wherein the residue at site 4L has been substituted.

35. The antibody of claim **30** wherein the residue at site 38L has been substituted.

36. The antibody of claim **30** wherein the residue at site 43L has been substituted.

37. The antibody of claim **30** wherein the residue at site 44L has been substituted.

38. The antibody of claim **30** wherein the residue at site 46L has been substituted.

39. The antibody of claim **30** wherein the residue at site 58L has been substituted.

40. The antibody of claim **30** wherein the residue at site 62L has been substituted.

41. The antibody of claim **30** wherein the residue at site 65L has been substituted.

42. The antibody of claim **30** wherein the residue at site 66L has been substituted.

43. The antibody of claim **30** wherein the residue at site 67L has been substituted.

44. The antibody of claim **30** wherein the residue at site 68L has been substituted.

45. The antibody of claim **30** wherein the residue at site 69L has been substituted.

46. The antibody of claim **30** wherein the residue at site 73L has been substituted.

47. The antibody of claim **30** wherein the residue at site 85L has been substituted.

48. The antibody of claim **30** wherein the residue at site 98L has been substituted.

49. The antibody of claim **30** wherein the residue at site 2H has been substituted.

50. The antibody of claim **30** wherein the residue at site 4H has been substituted.

51. The antibody of claim **30** wherein the residue at site 36H has been substituted.

52. The antibody of claim **30** wherein the residue at site 39H has been substituted.

53. The antibody of claim **30** wherein the residue at site 43H has been substituted.

54. The antibody of claim **30** wherein the residue at site 45H has been substituted.

55. The antibody of claim **30** wherein the residue at site 69H has been substituted.

56. The antibody of claim **30** wherein the residue at site 70H has been substituted.

57. The antibody of claim **30** wherein the residue at site 74H has been substituted.

58. The antibody of claim **30** wherein the residue at site 75H has been substituted.

59. The antibody of claim **30** wherein the residue at site 76H has been substituted.

60. The antibody of claim **30** wherein the residue at site 78H has been substituted.

61. The antibody of claim **30** wherein the residue at site 92H has been substituted.

62. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a consensus human variable domain, and further comprising an amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

63. A humanized antibody which lacks immunogenicity compared to a non-human parent antibody upon repeated administration to a human patient in order to treat a chronic disease in that patient, wherein the humanized antibody comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprises an amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

64. A humanized variant of a non-human parent antibody which binds an antigen and comprises a human variable domain comprising the most frequently occurring amino acid residues at each location in all human immunoglobulins of a human heavy chain immunoglobulin subgroup wherein amino acid residues forming Complementarity Determining Regions (CDRs) thereof comprise non-human antibody amino acid residues, and further comprises a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) introduces a glycosylation site which affects the antigen binding or affinity of the antibody; or (d) participates in the V_L–V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another.

65. The humanized variant of claim **63** which binds the antigen up to 3-fold more in the binding affinity than the parent antibody binds antigen.

66. A humanized antibody heavy chain variable domain comprising non-human Complementarity Determining

Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 24H, 73H, 76H, 78H, and 93H, utilizing the numbering system set forth in Kabat.

67. The humanized variable domain of claim **66** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

68. The humanized variable domain of claim **66** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

69. The humanized variable domain of claim **66** wherein the human antibody variable domain is a consensus human variable domain.

70. The humanized variable domain of claim **66** wherein the residue at site 24H has been substituted.

71. The humanized variable domain of claim **66** wherein the residue at site 73H has been substituted.

72. The humanized variable domain of claim **66** wherein the residue at site 76H has been substituted.

73. The humanized variable domain of claim **66** wherein the residue at site 78H has been substituted.

74. The humanized variable domain of claim **66** wherein the residue at site 93H has been substituted.

75. The humanized variable domain of claim **66** which further comprises an amino acid substitution at site 71H.

76. The humanized variable domain of claim **66** which further comprises amino acid substitutions at sites 71H and 73H.

77. The humanized variable domain of claim **66** which further comprises amino acid substitutions at sites 71H, 73H and 78H.

78. An antibody comprising the humanized variable domain of claim **66**.

79. A humanized variant of a non-human parent antibody which binds an antigen, wherein the humanized variant comprises Complementarity Determining Region (CDR) amino acid residues of the non-human parent antibody incorporated into a human antibody variable domain, and further comprises Framework Region (FR) substitutions at heavy chain positions 71H, 73H, 78H and 93H, utilizing the numbering system set forth in Kabat.

80. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution where the substituted FR residue:

(a) noncovalently binds antigen directly;

(b) interacts with a CDR; or

(c) participates in the V_L–V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another, and wherein the substituted FR residue is at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 24H, 36H, 39H, 43H, 45H, 69H, 70H, 73H, 74H, 76H, 78H, 92H and 93H, utilizing the numbering system set forth in Kabat.

81. The humanized variable domain of claim **80** wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

82. The humanized variable domain of claim **80** wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,407,213 B1
DATED : June 18, 2002
INVENTOR(S) : Carter et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 88,
Line 63, please delete "63" and insert therefor -- 79 --.

Signed and Sealed this

Third Day of December, 2002

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

EXHIBIT D

US007846441B1

(12) **United States Patent**
Hellmann

(10) **Patent No.: US 7,846,441 B1**
(45) **Date of Patent: Dec. 7, 2010**

(54) **TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventor: **Susan D. Hellmann**, San Carlos, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/208,649**

(22) Filed: **Dec. 10, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/069,346, filed on Dec. 12, 1997.

(51) **Int. Cl.**
A61K 39/395 (2006.01)

(52) **U.S. Cl.** **424/143.1**; 424/130.1; 424/133.1; 424/135.1; 424/136.1; 424/141.1; 424/142.1; 424/152.1; 424/155.1; 424/156.1; 424/172.1; 424/174.1

(58) **Field of Classification Search** 424/137.1, 424/138.1, 141.1, 227.1, 152.1, 130.1, 133.1, 424/135.1, 136.1, 142.1, 143.1, 155.1, 156.1, 424/172.1, 174.1
See application file for complete search history.

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(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

14 Claims, 2 Drawing Sheets

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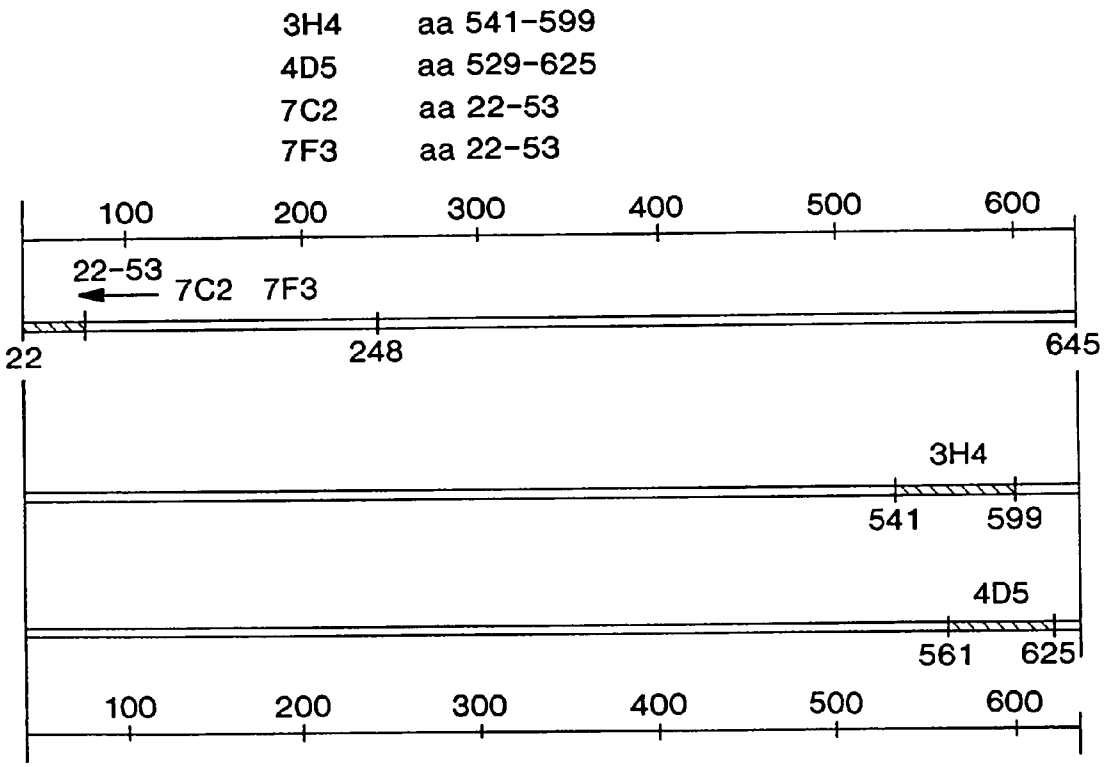
FIG. 1



3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR

541 599

4D5 epitope (SEQ ID NO:9) (64 residues)

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP

561 625

1 MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPA
38 SPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFL
75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186 TNRSRA

FIG. 2

TREATMENT WITH ANTI-ERBB2 ANTIBODIES

This is a non-provisional application claiming priority to provisional application No. 60/069,346, filed Dec. 12, 1997, the entire disclosure of which is hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237: 178-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al. *PNAS* (*USA*) 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize $p185^{erbB2}$-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (*USA*) 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2 or HERCEPTIN®) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]).

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198: 165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Suppl 2):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the treatment of disorders characterized by overexpression of ErbB2, and is based on the recognition that while treatment with anti-ErbB2 antibodies markedly enhances the clinical benefit of the use of chemotherapeutic agents in general, a syndrome of myocardial dysfunction that has been observed as a side-effect of anthracycline derivatives is increased by the administration of anti-ErbB2 antibodies.

Accordingly, the invention concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline derivative, e.g. doxorubicin or epirubicin, in the absence of an anthracycline derivative, to the human patient.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Although an antiproliferative effect is sufficient, in a preferred embodiment, the anti-ErbB2 antibody is capable of inducing cell death or is capable of inducing apoptosis. Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and

electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO:1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2" "c-Erb-B2" and "HER2" when used herein refer to the human protein and "her2", "erbB2" and "c-erb-B2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al, *PNAS* (*USA*) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the "PI uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science,* 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.,* 14(3):1909-1919 (1994); and Marchionni et al., *Nature,* 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177\text{-}244}$).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of

the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20): 14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a n-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the n-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. I, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al. *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the

techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al, *Proc. Natl. Acad. Sci. USA,* 81; 6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature,* 321:522-525 (1986); Reichmann et al, *Nature,* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.,* 2:593-596 (1992). The humanized antibody includes a PRIMATIZEDT™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I^{131}, I^{125}, Y^{90} and Re^{186}), chemo-

therapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (Taxotere®, Rhône-Poulenc Rorer, Antony, France), methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, caminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

II. Production of Anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al. *PNAS* (*USA*) 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc.*

Natl. Acad. Sci. USA, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity

chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5): 1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate

MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), *momordica charantia* inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA,* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-de-

rivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature,* 312: 604-608 [1984]).

(xi) Antibody-Salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatogra-

phy, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent, provided that the cytotoxic agent is other than an anthracycline derivative, e.g. doxorubicin, or epirubicin. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

IV. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

The treatment of the present invention involved the combined administration of an anti-ErbB2 antibody and a chemotherapeutic agent, other than an anthracycline derivative. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial factor (VEGF). Alternatively, or in addition, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. In a preferred embodiment, the ErbB2 antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Further information about suitable dosages is provided in the Example below.

V. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package inserts with instructions for use, including a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin, or epirubicin.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Va. 20110-2209 (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12226	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Example.

Example

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 $IgG_1\kappa$ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2-3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al. *Proc. Natl. Acad. Sci.* (*USA*) 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN®). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for p185" (Dillohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of $p185^{HER2}$, induces antibody-dependent

cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

Metastatic breast cancer

Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for $p185^{HER2}$].

Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs.

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

The ability to understand and willingness to sign a written informed consent form

Women≧18 years

Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

Prior cytotoxic chemotherapy for metastatic breast cancer

Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.

Concomitant malignancy that has not been curatively treated

A performance status of <60% on the Karnofsky scale

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regiments for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H_2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed. 2), New York, N.Y., Wiley, 1981, pp 13-17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN®, without increase in overall severe adverse events (AE):

	Enrolled	TTP(months)	RR(%)	AE(%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p <0.001 by log-rank test
**p <0.01 by X^2 test
CRx: chemotherapy
AC: anthracycline/cyclophosphamide treatment
H: HERCEPTIN ®
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade 3/4) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor the combined treatment with HERCEPTIN® and paclitaxel (TAXOL).

The disclosures of all citations in the specification are expressly incorporated herein by reference.

SEQUENCE LISTING

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                50                  55                  60

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                65                  70                  75

Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
                80                  85                  90

Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
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Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
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Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
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35 40 45

Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu
50 55 60

Gly Ala Cys Gln Pro
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The invention claimed is:

1. A method for the treatment of a human patient with a malignant progressing tumor or cancer characterized by overexpression of ErbB2 receptor, comprising administering a combination of an intact antibody which binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

2. The method of claim **1** wherein said patient has a malignant tumor.

3. The method of claim **1** wherein said patient has cancer.

4. The method of claim **3** wherein said cancer is selected from the group consisting of breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

5. The method of claim **4** wherein said cancer is breast cancer.

6. The method of claim **5** wherein said cancer is metastatic breast carcinoma.

7. The method of claim **1** wherein said antibody is a humanized 4D5 anti-ErbB2 antibody.

8. The method of claim **1** wherein said taxoid is paclitaxel.

9. The method of claim **8** wherein the effective amount of said combination is lower than the sum of the effective amounts of said anti-ErbB2 antibody and said taxoid, when administered individually, as single agents.

10. The method of claim **1** wherein efficacy is further measured by determining the response rate.

11. A method for the treatment of a human patient with ErbB2 overexpressing progressing metastatic breast cancer, comprising administering a combination of a humanized 4D5 anti-ErbB2 antibody and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

12. The method of claim **11** wherein said taxoid is paclitaxel.

13. A method for the treatment of a human patient with a progressing malignant tumor or cancer characterized by over-expression of ErbB2 receptor, comprising administering a combination of a humanized 4D5 anti-ErbB2 antibody which comprises a human Fc region and that binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

14. A method for the treatment of a human patient with ErbB2 expressing progressing metastatic breast cancer, comprising administering a combination of an antibody which binds to epitope 4D5 within the extracellular domain sequence and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

* * * * *

EXHIBIT E

US007892549B2

(12) **United States Patent**
Paton et al.

(10) **Patent No.: US 7,892,549 B2**
(45) **Date of Patent: *Feb. 22, 2011**

(54) **TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventors: **Virginia E. Paton**, Oakland, CA (US); **Steven Shak**, Burlingame, CA (US); **Susan D. Hellmann**, San Carlos, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1827 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **10/356,824**

(22) Filed: **Feb. 3, 2003**

(65) **Prior Publication Data**

US 2004/0037823 A9 Feb. 26, 2004

Related U.S. Application Data

(63) Continuation of application No. 09/208,649, filed on Dec. 10, 1998.

(60) Provisional application No. 60/069,346, filed on Dec. 12, 1997.

(51) **Int. Cl.**
A61K 39/395 (2006.01)
C07K 16/28 (2006.01)
C07K 16/30 (2006.01)

(52) **U.S. Cl.** **424/143.1**; 424/130.1; 424/133.1; 424/134.1; 424/135.1; 424/136.1; 424/138.1; 424/141.1; 424/152.1; 424/155.1; 424/156.1; 424/172.1; 424/174.1

(58) **Field of Classification Search** 424/130.1, 424/133.1, 138.1, 141.1, 143.1, 155.1, 174.1, 424/134.1, 135.1, 136.1, 152.1, 156.1, 172.1
See application file for complete search history.

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(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

17 Claims, 1 Drawing Sheet

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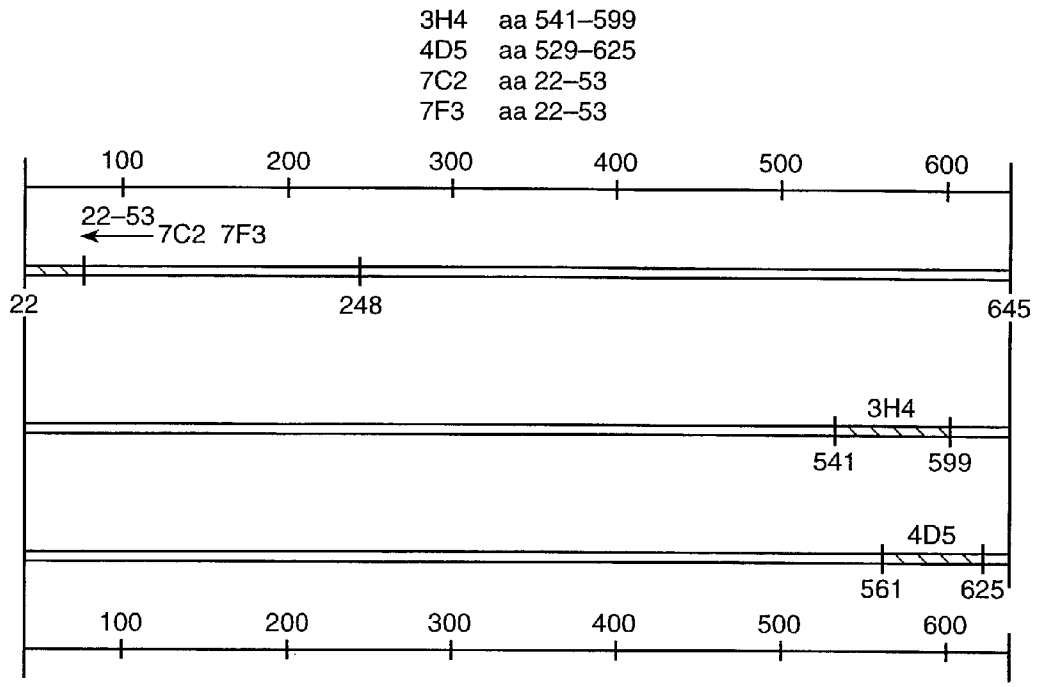

3H4 epitope (SEQ ID NO:8)

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR
541 **599**

4D5 epitope (SEQ ID NO:9)

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP
561 **625**

FIG._1

1 MELAALCRWGLLLALLPPGAA**STQVCTGTDMKLRLPA**
38 **SPETHLDMLRHLYQGC**QVVQGNLELTYLPTNASLSFL
75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186 TNRSRA

FIG._2

TREATMENT WITH ANTI-ERBB2 ANTIBODIES

This is a continuation of non-provisional application Ser. No. 09/208,649, filed Dec. 10, 1998, which claims priority under 35 USC §119 to provisional application No. 60/069,346, filed Dec. 12, 1997, the entire disclosures of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712[1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci.* USA 84:7159-7163 [1987]; DiFiore et al., *Science* 237:178-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B 104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al *PNAS* (USA) 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects

of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize $p185^{erbB2}$-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al In Vitro 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Nail Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369[1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (USA) 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of

this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2 or HERCEPTIN®) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]).

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198: 165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Suppl 2):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the treatment of disorders characterized by overexpression of ErbB2, and is based on the recognition that while treatment with anti-ErbB2 antibodies markedly enhances the clinical benefit of the use of chemotherapeutic agents in general, a syndrome of myocardial dysfunction that has been observed as a side-effect of anthracycline derivatives is increased by the administration of anti-ErbB2 antibodies.

Accordingly, the invention concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline derivative, e.g. doxorubicin or epirubicin, in the absence of an anthracycline derivative, to the human patient.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Although an antiproliferative effect is sufficient, in a preferred embodiment, the anti-ErbB2 antibody is capable of inducing cell death or is capable of inducing apoptosis. Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant

and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO: 1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2" "c-Erb-B2" and "HER2" when used herein refer to the human protein and "her2", "erbB2" and "c-erb-B2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS* (USA) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce P1 uptake in the "P1 uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science,* 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.,* 14(3):1909-1919 (1994); and Marchionni et al., *Nature,* 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177-244}$).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20): 14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. 1, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al. *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies"

may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci*. USA, 81:6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature,* 321:522-525 (1986); Reichmann et al., *Nature,* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.,* 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci*. USA, 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemo-

therapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France), methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce GI arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest GI also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1a, IL-2, IL-3, IL-4, IL-5, L-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactamcontaining prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

II. Production of anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al. *PNAS* (USA) 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R_1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Pluckthun, *Immunol. Revs.,* 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554(1990). Clackson et al., *Nature,* 352:624-628(1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc.*

Natl Acad. Sci. USA, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525(1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "bestfit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci*. USA, 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci*. USA, 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity

chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli,* which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175:217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., Proc. *Natl. Acad. Sci.* USA, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147:60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate

MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp. Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MXDTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci.* USA, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci.* USA, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-de-

rivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO. 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328:457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature,* 312:604-608 [1984]).

(xi) Antibody-salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s)(5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatogra-

phy, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immnol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent, provided that the cytotoxic agent is other than an anthracycline derivative, e.g. doxorubicin, or epirubicin. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

IV. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

The treatment of the present invention involved the combined administration of an anti-ErbB2 antibody and a chemotherapeutic agent, other than an anthracycline derivative. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial factor (VEGF). Alternatively, or in addition, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. In a preferred embodiment, the ErbB2 antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Further information about suitable dosages is provided in the Example below.

V. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package inserts with instructions for use, including a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin, or epirubicin.

DEPOSIT OF MATERIALS

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Example.

EXAMPLE

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 IgG_1K murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 5o:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3HER2-3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al. *Proc. Natl. Acad. Sci.* (USA) 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse mycloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN®). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci.* USA 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for $p185^{HER2}$ (Dillohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that con-

tain high levels of $p185^{HER2}$, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligihility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

Metastatic breast cancer

Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for $p185^{HER2}$].

Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

The ability to understand and willingness to sign a written informed consent form

Women≧18 years

Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

Prior cytotoxic chemotherapy for metastatic breast cancer

Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.

Concomitant malignancy that has not been curatively treated

A performance status of <60% on the Karnofsky scale

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+HER2 overexpression, or the metastatic site must have 2+ to 3+HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regiments for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed.2), New York, N.Y., Wiley, 1981, pp 13-17).

RESULTS

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN®, without increase in overall severe adverse events (AE):

	Enrolled	TTP(months)	RR(%)	AE(%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p < 0.001 by log-rank test
**p < 0.001 by X^2 test
CRx: chemotherapy
AC: anthracycline/cyclophosphamide treatment
H: HERCEPTIN ®
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade ¾) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor the combined treatment with HERCEPTIN® and paclitaxel (TAXOL®).

The disclosures of all citations in the specification are expressly incorporated herein by reference.

SEQUENCE LISTING

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Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu
                50                  55                  60

Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg
                65                  70                  75

Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
                80                  85                  90

Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
                95                 100                 105

Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
               110                 115                 120

Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
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Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
35 40 45

Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu
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Gly Ala Cys Gln Pro
65

The invention claimed is:

1. A method for the treatment of a human patient with breast cancer that overexpresses ErbB2 receptor, comprising administering a combination of an antibody that binds ErbB2, a taxoid, and a further growth inhibitory agent to the human patient in an amount effective to extend the time to disease progression in the human patient, wherein the antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

2. The method of claim **1** wherein the antibody is a humanized 4D5 anti-ErbB2 antibody.

3. The method of claim **1** wherein the antibody cross-blocks binding of 4D5 to the ErbB2 extracellular domain sequence.

4. The method of claim **1** wherein the antibody binds to amino acid residues in the region from about residue 529 to about residue 625 of the ErbB2 extracellular domain sequence.

5. A method for the treatment of a human patient with breast cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of a combination of an anti-ErbB2 antibody which binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid, and a further therapeutic agent, to the human patient.

6. The method of claim **5** wherein the breast cancer is metastatic breast carcinoma.

7. The method of claim **5** wherein the antibody is a humanized 4D5 anti-ErbB2 antibody.

8. The method of claim **7** wherein the antibody is administered as a 4 mg/kg dose and then weekly administration of 2 mg/kg.

9. The method of claim **5** wherein the taxoid is paclitaxel.

10. The method of claim **5** wherein efficacy is measured by determining the time to disease progression or the response rate.

11. The method of claim **5**, wherein the further therapeutic agent is selected from the group consisting of: another ErbB2 antibody, EGFR antibody, ErbB3 antibody, ErbB4 antibody, vascular endothelial growth factor (VEGF) antibody, cytokine, and growth inhibitory agent.

12. The method of claim **5** wherein the further therapeutic agent is another ErbB2 antibody.

13. The method of claim **5** wherein the further therapeutic agent is a vascular endothelial growth factor (VEGF) antibody.

14. The method of claim **5** wherein the further therapeutic agent is a growth inhibitory agent.

15. The method of claim **14** wherein the growth inhibitory agent is a DNA alkylating agent.

16. A method for the treatment of a human patient with ErbB2 overexpressing breast cancer, comprising administering a combination of an antibody that binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid and a further growth inhibitory agent, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in the human patient.

17. The method of claim **16** wherein the breast cancer is metastatic breast carcinoma.

* * * * *

EXHIBIT F

US008425908B2

(12) **United States Patent**
Hellmann

(10) **Patent No.: US 8,425,908 B2**
(45) **Date of Patent: Apr. 23, 2013**

(54) **TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventor: **Susan D. Hellmann**, San Carlos, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/185,329**

(22) Filed: **Jul. 18, 2011**

(65) **Prior Publication Data**

US 2012/0034213 A1 Feb. 9, 2012

Related U.S. Application Data

(60) Continuation of application No. 11/780,640, filed on Jul. 20, 2007, now Pat. No. 8,075,892, which is a division of application No. 10/909,998, filed on Aug. 2, 2004, which is a continuation of application No. 09/209,023, filed on Dec. 10, 1998, now abandoned.

(60) Provisional application No. 60/069,346, filed on Dec. 12, 1997.

(51) **Int. Cl.**

A61K 39/395	(2006.01)
C07K 14/75	(2006.01)
C07K 16/28	(2006.01)

(52) **U.S. Cl.**

USPC **424/143.1**; 424/130.1; 424/133.1; 424/134.1; 424/135.1; 424/136.1; 424/138.1; 424/141.1; 424/142.1; 424/155.1; 424/174.1

(58) **Field of Classification Search** 424/130.1, 424/133.1, 134.1, 135.1, 136.1, 138.1, 141.1, 424/142.1, 143.1, 155.1, 174.1

See application file for complete search history.

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Primary Examiner — Misook Yu
Assistant Examiner — Anne Holleran
(74) *Attorney, Agent, or Firm* — Diane Marschang; Ginger R. Dreger; Arnold & Porter LLP

(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The invention further provides a method of treating cancer in a human patient comprising administering effective amounts of an anti-ErbB2 antibody and a cardioprotectant to the patient.

7 Claims, 1 Drawing Sheet

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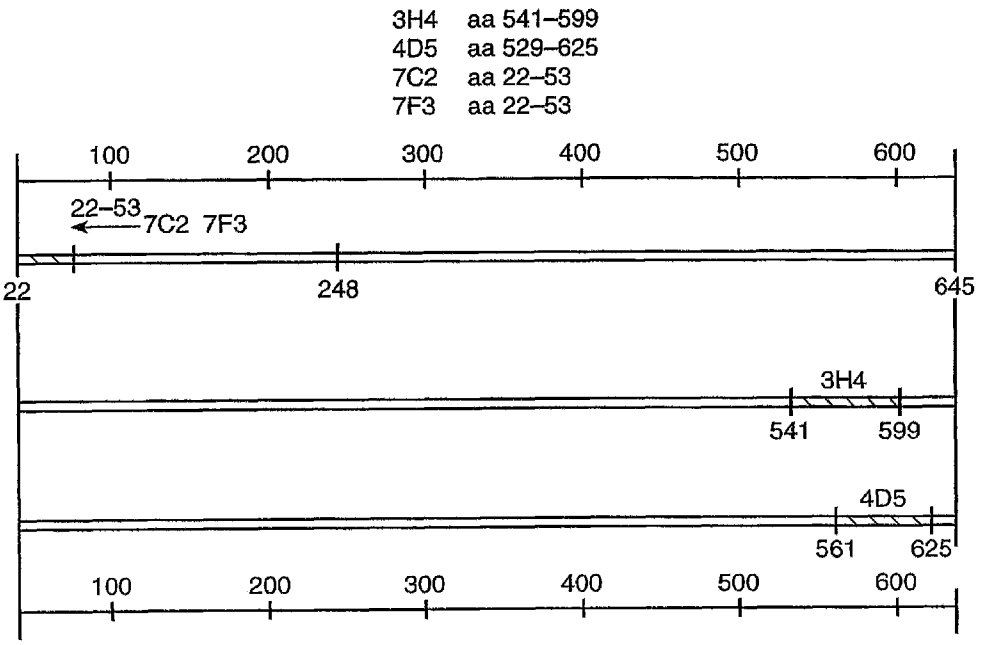

3H4 epitope (SEQ ID NO:8)

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR
541 **599**

4D5 epitope (SEQ ID NO:9)

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP
561 **625**

FIG._1

1 MELAALCRWGLLLALLPPGAA**STQVCTGTDMKLRLPA**
38 **SPETHLDMLRHLYQGC**QVVQGNLELTYLPTNASLSFL
75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186 TNRSRA

FIG._2

TREATMENT WITH ANTI-ERBB2 ANTIBODIES

This application is a continuation of U.S. application Ser. No. 11/780,640, filed Jul. 20, 2007, now U.S. Pat. No. 8,075,892 which application is a divisional of U.S. application Ser. No. 10/909,998, filed Aug. 2, 2004, which is a continuation of U.S. application Ser. No. 09/209,023, filed Dec. 10, 1998 (now abandoned) which claims priority under 35 U.S.C. Section 119(e) and the benefit of U.S. Provisional Application Ser. No. 60/069,346, filed Dec. 12, 1997, the entire disclosures of which are hereby incorporated by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Sep. 30, 2011, is named GNE0329C.txt and is 5,761 bytes in size.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The invention further provides a method of treating cancer in a human patient comprising administering effective amounts of an anti-ErbB2 antibody and a cardioprotectant to the patient.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237:178-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al. *PNAS* (*USA*) 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize $p185^{erbB2}$-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. In Vitro 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (*USA*) 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of

murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2 or HERCEPTIN®) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]).

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198: 165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Suppl 2):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the treatment of disorders characterized by overexpression of ErbB2, and is based on the recognition that while treatment with anti-ErbB2 antibodies markedly enhances the clinical benefit of the use of chemotherapeutic agents in general, a syndrome of myocardial dysfunction that has been observed as a side-effect of anthracycline derivatives is increased by the administration of anti-ErbB2 antibodies.

Accordingly, the invention concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline derivative, e.g. doxorubicin or epirubicin, in the absence of an anthracycline derivative, to the human patient.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Although an antiproliferative effect is sufficient, in a preferred embodiment, the anti-ErbB2 antibody is capable of inducing cell death or is capable of inducing apoptosis. Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition.

In a further embodiment, the invention provides a method of treating ErbB2 expressing cancer in a human patient comprising administering effective amounts of an anti-ErbB2 antibody and a cardioprotectant to the patient. The cancer is preferably characterized by overexpression of ErbB2. In one embodiment, the method further comprises administering an anthracyline antibiotic to the patient. In another embodiment, an anthracycline antibiotic is not administered to the patient

with the anti-ErbB2 antibody or cardioprotectant. One or more additional chemotherapeutic agents may also be administered to the patient.

The invention further provides an article of manufacture comprising a container, a composition within the container comprising an anti-ErbB2 antibody and a package insert instructing the user of the composition to administer the anti-ErbB2 antibody composition and a cardioprotectant to a patient.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO: 1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2). FIG. **2** discloses the full length sequence as SEQ ID NO: 10.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2", "c-Erb-B2" and "HER2" when used herein refer to the human protein and "her2", "erbB2" and "c-erb-B2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS* (*USA*) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells.

Preferred cell death-inducing antibodies are those which induce PI uptake in the "PI uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most

preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science,* 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.,* 14(3):1909-1919 (1994); and Marchionni et al., *Nature,* 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177-244}$).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20): 14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. I, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al. *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature,* 321:522-525 (1986); Reichmann et al., *Nature,* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.,* 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

An "ErbB2-expressing cancer" is one comprising cells which have ErbB2 protein present at their cell surface, such that an anti-ErbB2 antibody is able to bind to the cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; antimetabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; carminomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as antiestrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone; and antiandrogens such as flutamide and nilutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione. Other anthracycline antibiotics include

epirubicin, daunorubicin, carminomycin, detorubicin, esorubicin, marcellomycin, quelamycin, rodorubicin, idarubicin, as well as pharmaceutically active salts, acids or derivatives of any of these.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions,* 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

A "cardioprotectant" is a compound or composition which prevents or reduces myocardial dysfunction (i.e. cardiomyopathy and/or congestive heart failure) associated with administration of a drug, such as an anthracycline antibiotic and/or an anti-ErbB2 antibody, to a patient. The cardioprotectant may, for example, block or reduce a free-radical-mediated cardiotoxic effect and/or prevent or reduce oxidative-stress injury. Examples of cardioprotectants encompassed by the present definition include the iron-chelating agent dexrazoxane (ICRF-187) (Seifert et al. *The Annals of Pharmacotherapy* 28:1063-1072 [1994]); a lipid-lowering agent and/or anti-oxidant such as probucol (Singal et al. *J. Mol. Cell Cardiol.* 27:1055-1063 [1995]); amifostine (aminothiol 2-[(3-aminopropyl)amino]ethanethiol-dihydrogen phosphate ester, also called WR-2721, and the dephosphorylated cellular uptake form thereof called WR-1065) and S-3-(3-methylaminopropylamino)propylphosphorothioic acid (WR-151327), see Green et al. *Cancer Research* 54:738-741 (1994); digoxin (Bristow, M. R. In: Bristow M R, ed. *Drug-Induced Heart Disease*. New York: Elsevier 191-215 [1980]); beta-blockers such as metoprolol (Hjalmarson et al. *Drugs* 47:Suppl 4:31-9 [1994]; and Shaddy et al. *Am. Heart J.* 129:197-9 [1995]); vitamin E; ascorbic acid (vitamin C); free radical scavengers such as oleanolic acid, ursolic acid and N-acetylcysteine (NAC); spin trapping compounds such as alpha-phenyl-tert-butyl nitrone (PBN); (Paracchini et al., *Anticancer Res.* 13:1607-1612 [1993]); selenoorganic compounds such as P251 (Elbesen); and the like.

II. Production of Anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al. *PNAS* (*USA*) 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuc-

cinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130: 151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348: 552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to

have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in W096/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow

cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODEFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang et al., *Proc. Natl Acad. Sci. USA,* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A che-

motherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature,* 312: 604-608 [1984]).

(xi) Antibody-Salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s)(5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse

isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine, growth inhibitory agent and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

IV. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelic, inflammatory, angiogenic and immunologic disorders.

The antibodies, chemotherapeutic agents and cardioprotectants of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB2 antibody and a chemotherapeutic agent, other than an anthracycline derivative. The present invention contemplates admin-

istration of cocktails of different chemotherapeutic agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial factor (VEGF). Alternatively, or in addition, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. In a preferred embodiment, the ErbB2 antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

The present invention also provides a method of treating ErbB2 expressing cancer, especially cancer characterized by overexpression of ErbB2, by administering effective amounts of an anti-ErbB2 antibody and a cardioprotectant to a patient susceptible to, or diagnosed with, cancer.

In one embodiment, the method further comprises administering one or more chemotherapeutic agents to the patient. For example, an anthracycline antibiotic may be administered to the patient along with the cardioprotectant and anti-ErbB2 antibody (as well as one or more other optional chemotherapeutic agents).

In an alternative embodiment, an anthracycline antibiotic is not administered to the patient with the anti-ErbB2 antibody or cardioprotectant. Thus, while the patient may have been exposed to an anthracycline antibiotic in a previous treatment regimen, in this embodiment of the invention, the therapeutic regimen does not entail the administration of an antracycline antibiotic (i.e. the method is performed in the absence of an anthracycline antibiotic). This embodiment of the invention contemplates however the optional administration of one or more other chemotherapeutic agents (other than anthracycline antibiotics) to the patient.

Combined administration of the anti-ErbB2 antibody and cardioprotectant includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Moreover, administration of the cardioprotectant may be delayed until the patient has been given a lifetime dose of anti-ErbB2 antibody and/or anthracycline antibiotic which is considered to result in a high risk of cardiac side effects (e.g. a lifetime doxorubicin dose of 350 mg/m^2). In one embodiment, the cardioprotectant is administered to the patient both prior to administration of the anti-ErbB2 antibody and concurrently therewith.

The effective amount of the cardioprotectant is that which reduces or prevents myocardial dysfunction (e.g. cardiomyopathy and/or congestive heart failure) resulting from administration of the anti-ErbB2 antibody (and optionally an anthracycline antibiotic or other chemotherapeutic agent) to the patient. Myocardial dysfunction can be assessed by a variety of different methods including physical examination, electrocardiography, echocardiography, angiocardiography, and endomyocardial biopsy (reviewed in Singal et al. *NEJM* 339:900-905 (1998), expressly incorporated herein by reference). The preferred method for determining myocardial dysfunction comprises measuring the ejection fraction by echocardiography or angiocardiography. Suitable dosages for the cardioprotectants dexrazoxane and probucol have been described, for example, in Seifert et al. *The Annals of Pharmacotherapy* 28:1063-1072 (1994) and Singal et al. *J. Mol. Cell Cardiol.* 27:1055-1063 (1995), respectively. The effective amount of the cardioprotectant may be determined based on the amount of anti-ErbB2 antibody and/or anthracycline antibiotic agent administered to the patient. Generally, the amount of cardioprotectant will exceed the amount of chemotherapeutic agent or anti-ErbB2 antibody causing the cardiotoxicity. For example, the ratio of cardioprotectant: antibody or chemotherapeutic agent may be from about 1:1 to about 100:1.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Further information about suitable dosages is provided in the Example below.

V. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in

the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package inserts with instructions for use, including for example a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin, or epirubicin, or instructing the user of the composition to administer the anti-ErbB2 antibody composition and a cardioprotectant to a patient.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Example.

Example

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 $IgG_1\kappa$ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2-3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al. *Proc. Natl. Acad. Sci. (USA)* 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody) (HERCEPTIN®). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for $p185^{HER2}$ (Dillohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of $p185^{HER2}$, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

- Metastatic breast cancer
- Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for $p185^{HER2}$].
- Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

- The ability to understand and willingness to sign a written informed consent form
- Women≧18 years
- Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

- Prior cytotoxic chemotherapy for metastatic breast cancer Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.
- Concomitant malignancy that has not been curatively treated
- A performance status of <60% on the Karnofsky scale
- Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator
- Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)
- Use of investigational or unlicensed agents within 30 days prior to study entry
- Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regiments for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H_2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed. 2), New York, N.Y., Wiley, 1981, pp 13-17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN®, without increase in overall severe adverse events (AE):

	Enrolled	TTP (months)	RR (%)	AE (%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*$p < 0.001$ by log-rank test
**$p < 0.01$ by X^2 test
CRx: chemotherapy
AC: anthracycline/cyclophosphamide treatment
H: HERCEPTIN ®
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade 3/4) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor the combined treatment with HERCEPTIN® and paclitaxel (TAXOL®).

The disclosures of all citations in the specification are expressly incorporated herein by reference.

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Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg Ile Val Arg Gly Thr
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Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala Val Leu Asp Asn Gly Asp
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Pro Leu Asn Asn Thr Thr Pro Val Thr Gly Ala Ser Pro Gly Gly Leu
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Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
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Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
                85                  90                  95

Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
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Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
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Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
145                 150                 155                 160

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175

Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala
            180                 185                 190
```

What is claimed is:

1. A method for the treatment of a human patient with gastric cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of a combination of an anti-ErbB2 antibody, or an antigen-binding fragment thereof, and a chemotherapeutic agent, to the human patient, wherein the chemotherapeutic agent is capecitabine or 5-fluorouracil, wherein the attending physician is provided with instructions including a warning that the treatment is not to be performed in combination with an anthracycline derivative and wherein said antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

2. The method of claim **1** wherein the chemotherapeutic agent is capecitabine.

3. The method of claim **2** wherein said antibody is a humanized 4D5 anti-ErbB2 antibody, or an antigen-binding fragment thereof.

4. The method of claim **3** wherein the antibody is rhuMAb HER2.

5. The method of claim **1** further comprising the administration of a further chemotherapeutic agent.

6. The method of claim **5** wherein the further chemotherapeutic agent is cisplatin.

7. The method of claim **1** wherein efficacy is defined in terms of time to disease progression or response rate.

* * * * *

EXHIBIT G

US006627196B1

(12) **United States Patent**
Baughman et al.

(10) **Patent No.: US 6,627,196 B1**
(45) **Date of Patent: Sep. 30, 2003**

(54) **DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventors: **Sharon A. Baughman**, Ventura, CA (US); **Steven Shak**, Burlingame, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/648,067**

(22) Filed: **Aug. 25, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/213,822, filed on Jun. 23, 2000, and provisional application No. 60/151,018, filed on Aug. 27, 1999.

(51) **Int. Cl.**[7] **A61K 39/395**

(52) **U.S. Cl.** **424/138.1**; 424/131.1; 424/133.1; 424/134.1; 424/135.1; 424/136.1; 424/137.1; 424/139.1; 424/141.1; 424/142.1; 424/143.1; 424/144.1; 424/145.1; 424/146.1; 424/147.1; 424/150.1; 424/151.1; 424/152.1; 424/153.1; 424/154.1; 424/155.1; 424/156.1; 424/158.1; 424/172.1; 424/174.1

(58) **Field of Search** 424/130.1, 138.1, 424/141.1, 142.1, 152.1, 155.1, 131.1, 133.1, 134.1, 135.1, 136.1, 137.1, 139.1, 143.1, 144.1, 145.1, 146.1, 147.1, 150.1, 151.1, 153.1, 154.1, 156.1, 158.1, 172.1, 174.1

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(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with anti-ErbB2 antibody.

33 Claims, 5 Drawing Sheets

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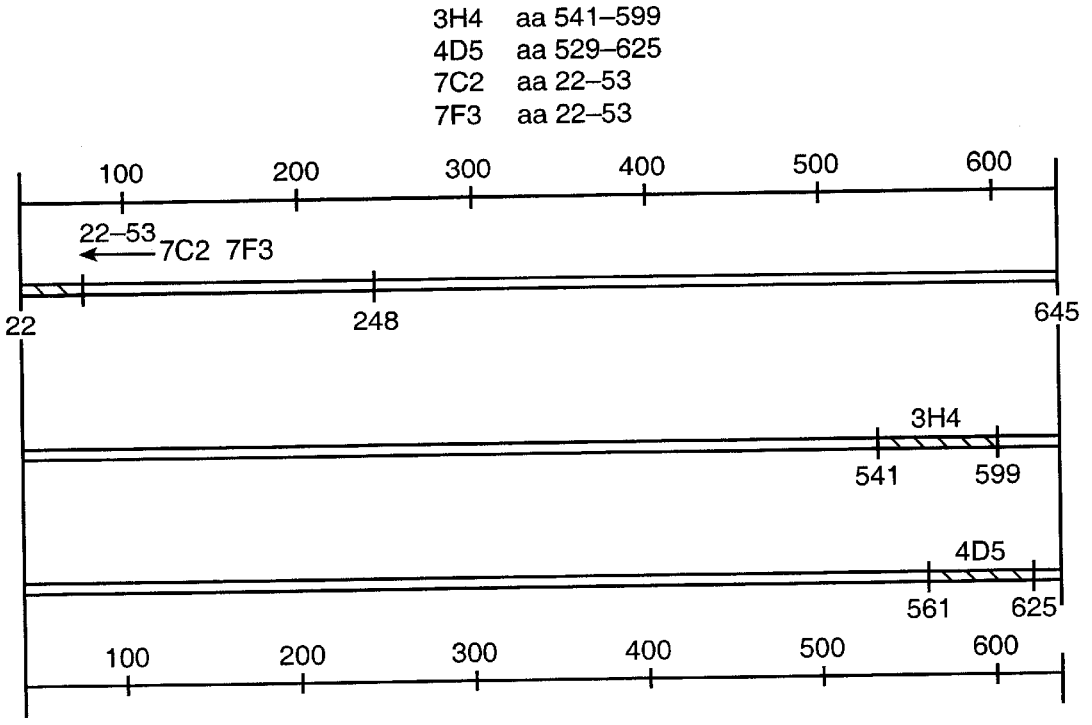

3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR

541 **599**

4D5 epitope (SEQ ID NO:9) 64 residues

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP

561 **625**

FIG._1

1 MELAALCRWGLLLALLPPGAA**STQVCTGTDMKLRLPA**

38 **SPETHLDMLRHLYQGC**QVVQGNLELTYLPTNASLSFL

75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN

112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI

149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID

186 TNRSRA

FIG._2

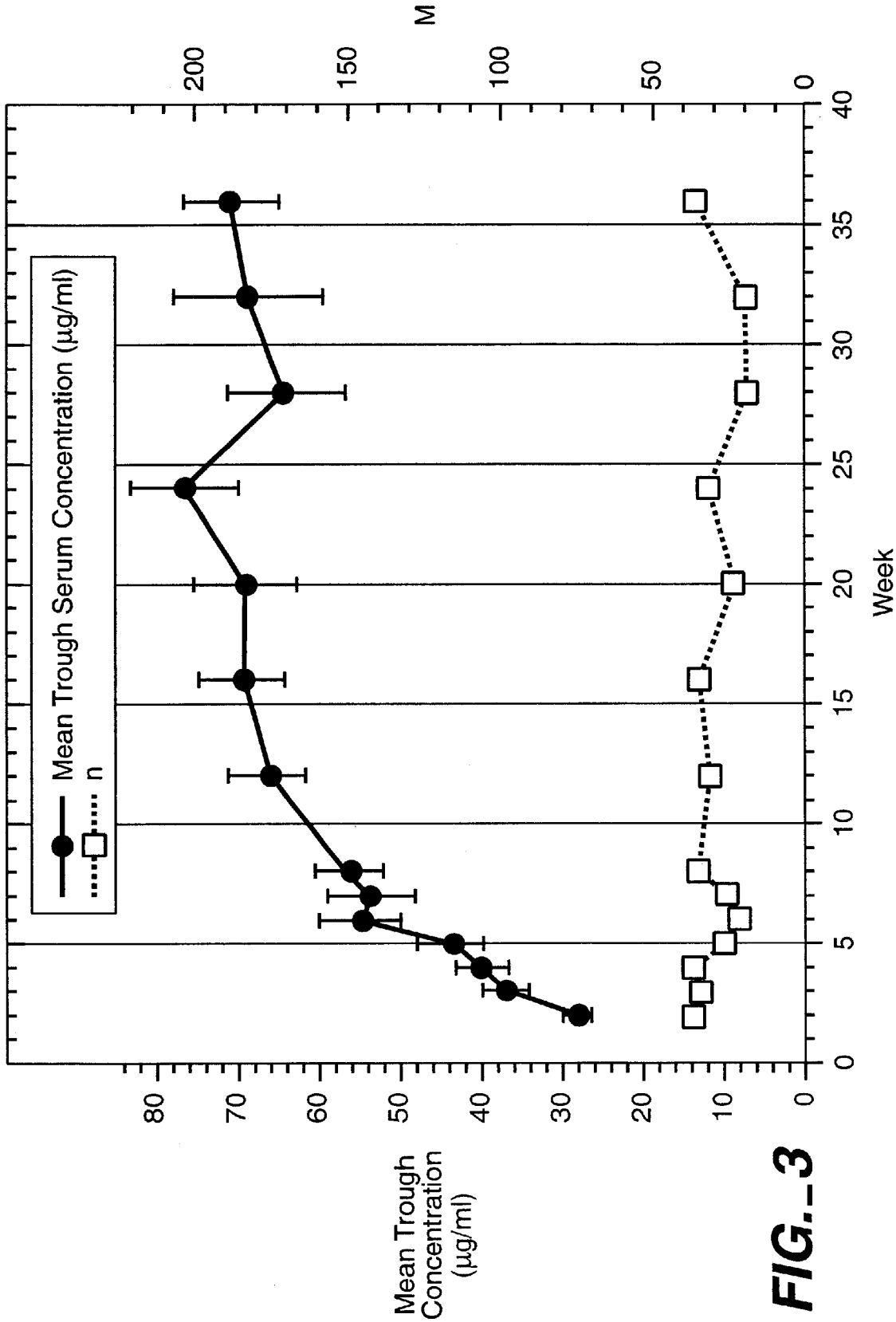
Mean Trough Serum Concentration (µg/ml)
n
Mean Trough Concentration (µg/ml)
M
Week
0
10
20
30
40
50
60
70
80
0
50
100
150
200
0
5
10
15
20
25
30
35
40

FIG._3

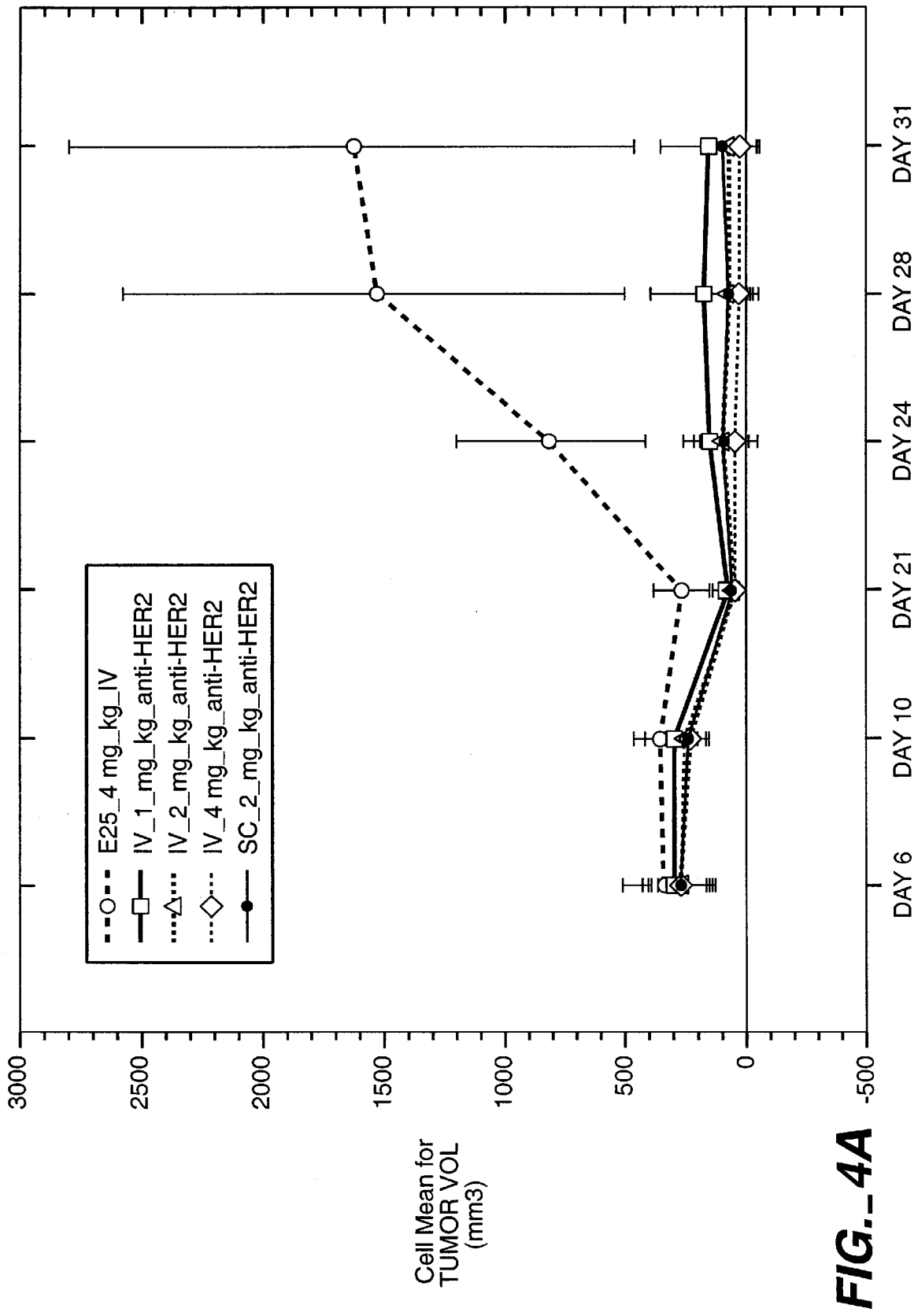
E25_4 mg_kg_IV
IV_1_mg_kg_anti-HER2
IV_2_mg_kg_anti-HER2
IV_4 mg_kg_anti-HER2
SC_2_mg_kg_anti-HER2
Cell Mean for TUMOR VOL (mm3)
3000
2500
2000
1500
1000
500
0
-500
DAY 6
DAY 10
DAY 21
DAY 24
DAY 28
DAY 31

FIG._4A

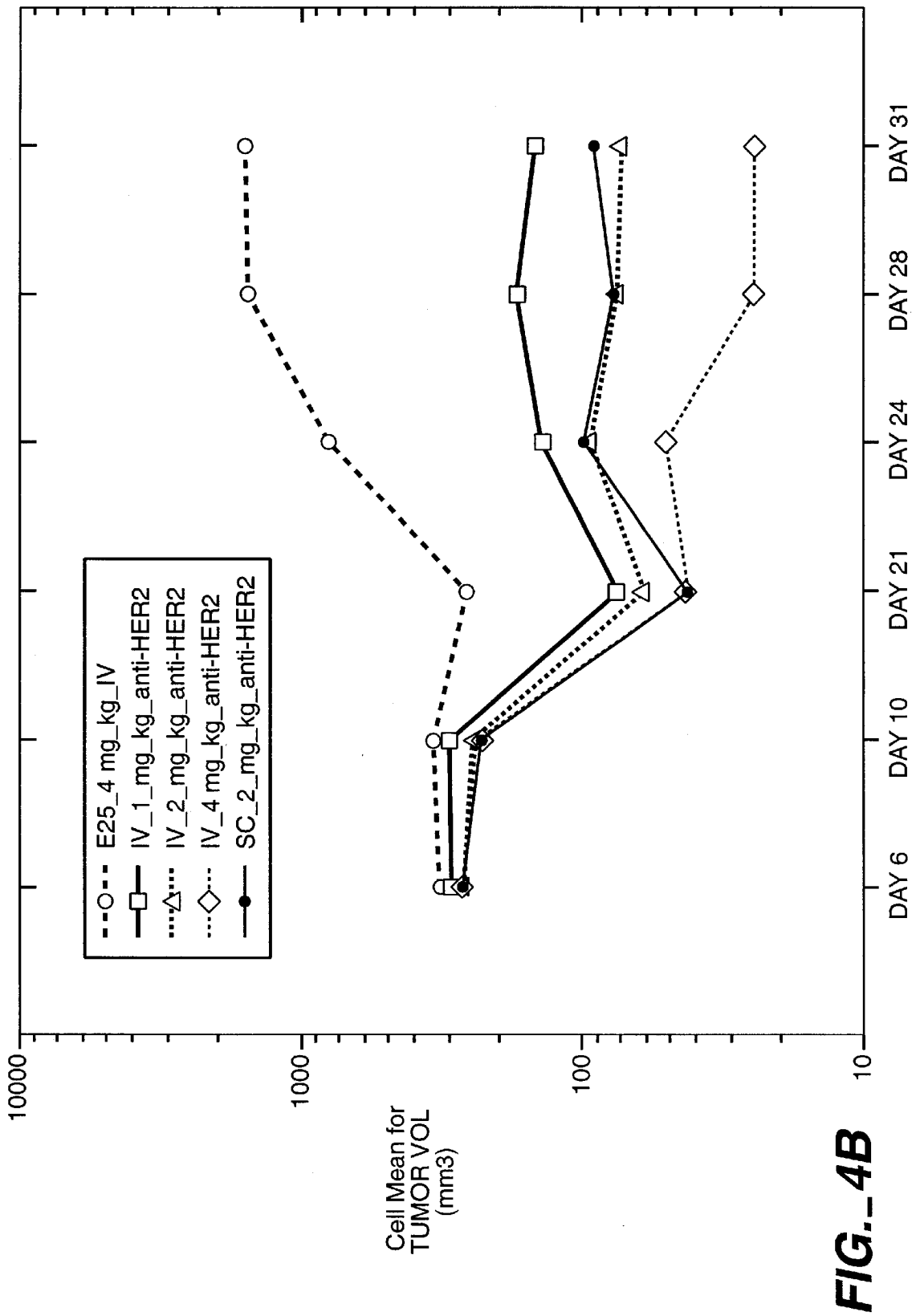
E25_4 mg_kg_IV
IV_1_mg_kg_anti-HER2
IV_2_mg_kg_anti-HER2
IV_4 mg_kg_anti-HER2
SC_2_mg_kg_anti-HER2
Cell Mean for TUMOR VOL (mm3)
10000
1000
100
10
DAY 6
DAY 10
DAY 21
DAY 24
DAY 28
DAY 31

FIG._4B

VARIABLE LIGHT

```
          1       10        20          30              40
2C4       DTVMTQSHKIMSTSVGDRVSITC [KASQDVSIGVA----] WYQQRP
           **    **** *      *                        *
574       DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA----] WYQQKP
                                   *   *  ****
hum kI    DIQMTQSPSSLSASVGDRVTITC [RASQSVSTSSYSYMH] WYQQKP

                    50          60        70        80
2C4       GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA
           **                   *  *          *    * *
574       GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISSLQP
                     *  ****
hum kI    GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP

                   90          100
2C4       EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:10)
            * *                  *   *
574       EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:12)
                      ***
hum kI    EDFATYYC [QQYNSLPYT] FGQGTKVEIK (SEQ ID NO:14)
```

FIG._5A

VARIABLE HEAVY

```
          1       10        20          30          40
2C4       EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS
              **  **  *  * ***  **                   *
574       EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA
                                       ** * *
humIII    EVQLVESGGGSVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

                    50        60          70        80
2C4       HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM
          *  *   **                     *** *    **** *
574       PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL
                  *  ******  ** ****       * * *
humIII    PGKGLEWVS [VISGDGGSTYYADSVKG] RFTISRDDSKNTLYL

                     90         100          110
2C4       ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLVTSS (SEQ ID NO:11)
          ***  **                              *
574       QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTLVTVSS (SEQ ID NO:13)
                            ** ***
humIII    QMNSLRAEDTAVYYCAR [GRGGGS--DY] WGQGTLVTVSS (SEQ ID NO:15)
```

FIG._5B

DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES

RELATED APPLICATIONS

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application No. 60/151,018, filed Aug. 27, 1999 and No. 60/213,822, filed Jun. 23, 2000, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2 or disorders expressing epidermal growth factor receptor (EGFR), comprising administering to a human or animal presenting the disorders a therapeutically effective amount of an antibody that binds ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 or expressing EGFR, where the treatment is with an anti-ErbB2 antibody administered by front loading the dose of antibody during treatment by intravenous and/or subcutaneous administration. The invention optionally includes treatment of cancer in a human patient with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, a taxoid. The taxoid may be, but is not limited to paclitaxel or docetaxel. The invention further includes treatment of cancer in a human patient with a combination of anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, an anthracycline derivative. Optionally, treatment with a combination of anti-ErbB2 and an anthracycline derivative includes treatment with an effective amount of a cardioprotectant. The present invention further concerns infrequent dosing of anti-ErbB2 antibodies.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177–182 [1987]; Slamon et al., *Science* 244:707–712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159–7163 [1987]; DiFiore et al., *Science* 237:78–182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578–10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695–706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) a inhibits colony formation of these cells. In Drebin et al. *PNAS* (*USA*) 83:9129–9133 (1986), the 7.16.4 antibody was shown to

inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387–394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273–277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277–290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165–1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel,including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize p 185 -overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550–1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72–82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117–127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979–986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255–263 (1993); Pietras et al. *Oncogene* 9:1829–1838 (1994); Vitetta et al. *Cancer Research* 54:5301–5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20): 14661–14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300–5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.*91:7202–7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933–937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543–548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361–5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350–362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (*USA*) 88:8691–8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580–2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth, whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress-ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et a. *Int. J. Cancer* 53:401–408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771–2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575–4580 (1991); Shawver et al. *Cancer Res.* 54:1367–1373 (1994); Arteaga et al. *Cancer Res.* 54:3758–3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160–15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2, HERCEPTIN®, or HERCEPTIN® anti-ErbB2 antibody) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin Oncol.* 14:737–744 [1996]). The recommended initial loading dose for HERCEPTIN®O is 4 mg/kg administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg and can be administered as a 30-minute infusion if the initial loading dose is well tolerated.

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], *supra*; Ravdin and Chamness, *Gene* 159:19–27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198:165–184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11 (3 Suppl 1):43–48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the discovery that an early attainment of an efficacious target trough serum concentration by providing an initial dose or doses of anti-ErbB2 antibodies followed by subsequent doses of equal or smaller amounts of antibody (greater front loading) is more efficacious than conventional treatments. The efficacious target trough serum concentration is reached in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The target serum concentration is thereafter maintained by the administration of maintenance doses of equal or smaller amounts for the remainder of the treatment regimen or until suppression of disease symptoms is achieved.

The invention further concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of an anti-ErbB2 antibody subcutaneously. Preferably, the initial dose (or doses) as well as the subsequent maintenance dose or doses are administered subcutaneously. Optionally, where the patient's tolerance to the anti-ErbB2 antibody is unknown, the initial dose is administered by intravenous infusion, followed by subcutaneous administration of the maintenance doses if the patient's tolerance for the antibody is acceptable.

According to the invention, the method of treatment involves administration of an initial dose of anti-ErbB2 antibody of more than approximately 4 mg/kg, preferably more than approximately 5 mg/kg. The maximum initial dose or a subsequent dose does not exceed 50 mg/kg, preferably does not exceed 40 mg/kg, and more preferably does not exceed 30 mg/kg. Administration is by intravenous or subcutaneous administration, preferably intravenous infusion or bolus injection, or more preferably subcutaneous bolus injection. The initial dose may be one or more administrations of drug sufficient to reach the target trough serum concentration in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less.

According to the invention, the initial dose or doses is/are followed by subsequent doses of equal or smaller amounts of antibody at intervals sufficiently close to maintain the trough serum concentration of antibody at or above an efficacious target level. Preferably, an initial dose or subsequent dose does not exceed 50 mg/kg, and each subsequent dose is at least 0.01 mg/kg. Preferably the amount of drug

administered is sufficient to maintain the target trough serum concentration such that the interval between administration cycles is at least one week. Preferably the trough serum concentration does not exceed 2500 μg/ml and does not fall below 0.01 μg/ml during treatment. The front loading drug treatment method of the invention has the advantage of increased efficacy by reaching a target serum drug concentration early in treatment. The subcutaneous delivery of maintenance doses according to the invention has the advantage of being convenient for the patient and health care professionals, reducing time and costs for drug treatment. Preferably, the initial dose (or the last dose within an initial dose series) is separated in time from the first subsequent dose by 4 weeks or less, preferably 3 weeks or less, more preferably 3 weeks or less, most preferably 1 week or less.

In an embodiment of the invention, the initial dose of anti-ErbB2 is 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous administration, such as intravenous infusion or subcutaneous bolus injection. The subsequent maintenance doses are 2 mg/kg delivered once per week by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. The choice of delivery method for the initial and maintenance doses is made according to the ability of the animal or human patient to tolerate introduction of the antibody into the body. Where the antibody is well-tolerated, the time of infusion may be reduced. The choice of delivery method as disclosed for this embodiment applies to all drug delivery regimens contemplated according to the invention.

In another embodiment, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In still another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

In yet another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

In another embodiment, the invention includes initial doses of at least 1 mg/kg, preferably 4 mg/kg, anti-ErbB2 antibody on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In another embodiment, the invention includes an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

In still another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is 2–3 times per week for 3 weeks. In one embodiment of the invention, each dose is approximately 25 mg/kg or less for a human patient, preferably approximately 10 mg/kg or less. This 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

In another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The method of the invention may further comprise administration of a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind o the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form. Other preferred ErbB2-binding antibodies include, but are not limited to, antibodies 7C2, 7F3, and 2C4, preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

The present application also provides a method of therapy involving infrequent dosing of an anti-ErbB2 antibody. In particular, the invention provides a method for the treatment of cancer (e.g. cancer characterized by overexpression of the ErbB2 receptor) in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). For instance, the antibody may be administered about every three weeks, about two to about 20 times, e.g. about six times. The first dose and subsequent dose may each be from about 2 mg/kg to about 16 mg/kg; e.g. from about 4 mg/kg to about 12 mg/kg; and optionally from about 6 mg/kg to about 12 mg/kg. Generally, two or more subsequent doses (e.g. from about two to about ten subsequent doses) of the antibody are administered to the patient, and those subsequent doses are preferably separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). The two or more subsequent doses may each be from about 2 mg/kg to about 16 mg/kg; or from about 4 mg/kg to about 12 mg/kg; or from about 6 mg/kg to about 12 mg/kg. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, and a package insert containing instructions to administer the antibody according to such methods.

The presently described dosing protocols may be applied to other anti-ErbB antibodies such as anti-epidermal growth factor receptor (EGFR), anti-ErbB3 and anti-ErbB4 antibodies. Thus, the invention provides a method for the treatment of cancer in a human patient, comprising administering an effective amount of an anti-ErbB antibody to the human patient, the method comprising administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB antibody; and administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose. Alternatively, or additionally, the invention pertains to a method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an anti-ErbB antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two

weeks. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB antibody, and a package insert containing instructions to administer the antibody according to such methods.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition. According to the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody at an initial dose of 5 mg/kg followed by the same or smaller subsequent dose or doses. In another embodiment of the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody subcutaneously for at least one of the doses, preferably for all of the subsequent doses following the initial dose, most preferably for all doses.

In a further aspect, the invention provides a method of treating ErbB2 expressing cancer in a human patient comprising administering to the patient effective amounts of an anti-ErbB2 antibody and a chemotherapeutic agent. In one embodiment of the invention, the chemotherapeutic agent is a taxoid including, but not limited to, paclitaxel and docetaxel. In another embodiment, the chemotherapeutic agent is an anthracyline derivative including, but not limited to, doxorubicin or epirubicin. In still another embodiment of the invention, treatment with an anti-ErbB2 antibody and an anthracycline derivative further includes administration of a cardioprotectant to the patient. In still another embodiment, an anthracycline derivative is not administered to the patient with the anti-ErbB2 antibody. One or more additional chemotherapeutic agents may also be administered to the patient. The cancer is preferably characterized by overexpression of ErbB2.

The invention further provides an article of manufacture comprising a container, a composition within the container comprising an anti-ErbB2 antibody and a package insert instructing the user of the composition to administer the anti-ErbB2 antibody composition and a chemotherapeutic agent to a patient. In another embodiment, the chemotherapeutic agent is other than an anthracycline, and is preferably a taxoid, such as TAXOL®. In still another embodiment, the chemotherapeutic agent is an anthracycline, including but not limited to, doxorubicin or epirubicin. In yet another embodiment, the chemotherapeutic agent is an anthracycline and the package insert further instructs the user to administer a cardioprotectant.

The methods and compositions of the invention comprise an anti-ErbB2 antibody and include a humanized anti-ErbB2 antibody. Thus, the invention further pertains to a composition comprising an antibody that binds ErbB2 and the use of the antibody for treating ErbB2 expressing cancer, e.g., ErbB2 overexpressing cancer, in a human. The invention also pertains to the use of the antibody for treating EGFR expressing cancer. Preferably the antibody is a monoclonal antibody 4D5, e.g., humanized 4D5 (and preferably huMAb4D5–8 (HERCEPTIN® anti-ErbB2 antibody); or monoclonal antibody 2C4, e.g., humanized 2C4. The antibody may be an intact antibody (e.g., an intact IgG, antibody) or an antibody fragment (e.g., a Fab, $F(ab')_2$, diabody, and the like). The variable light chain and variable heavy chain regions of humanized anti-ErbB2 antibody 2C4 are shown in FIGS. **5**A and **5**B.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67 (10):6179–6191 [October 1993]; Renz et *J. Cell Biol.* 125(6):1395–1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 b bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 2–4 hours at 4° C. The complexes were precipitated, applied to a 10–20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO: 1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

FIG. **3** is a graph of anti-ErbB2 antibody (HERCEPTIN®) trough serum concentration (μg/ml, mean ±SE, dark circles) by week from week 2 through week 36 for ErbB2 overexpressing patients treated with HERCEPTIN® anti-ErbB2 antibody at 4 mg/kg initial dose, followed by 2 mg/kg weekly. The number of patients at each time point is represented by "n" (white squares).

FIG. **4**A is a linear plot of tumor volume changes over time in mice treated with HERCEPTIN® anti-ErbB2 antibody. FIG. **4**B is a semi-logarithmic plot of the same data as in FIG. **4**A such that the variation in tumor volume for the treated animals is observed more readily.

FIGS. **5**A and **5**B depict alignments of the amino acid sequences of the variable light (V_L)(FIG. **5**A) and variable heavy (V_H) (FIG. **5**B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 10 and 11, respectively); V_L and V_H domains of humanized Fab version 574 (SEQ ID Nos. 12 and 13, respectively), and human V_L and V_H consensus frameworks (hum κl, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 14 and 15, respectively). Asterisks identify differences between humanized Fab version 574 and murine monoclonal antibody 2C4 or between humanized Fab version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets. Humanized Fab version 574, with the changes ArgH71Val, AspH73Arg and IleH69Leu, appears to have binding restored to that of the original chimeric 2C4 Fab fragment. Additional FR and/or CDR residues, such as L2, L54, L55, L56, H35 and/or H48, may be modified (e.g. substituted as follows-IleL2Thr; ArgL54Leu; TyrL55Glu; ThrL56Ser; AspH35Ser; and ValH48Ile) in order to further refine or enhance binding of the humanized antibody. Alternatively, or additionally, the humanized antibody may be affinity matured in order to further improve or refine its affinity and/or other biological activities.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

An "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3 and ErbB4 receptors as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881–914 (1987), including variants thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS (USA)* 87:4207–4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL RB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.).

"ErbB3" and"HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS (USA)* 86:9193–9197 (1989), including variants thereof. Examples of antibodies which bind HER3 are described in U.S. Pat. No. 5,968,511 (Akita and Sliwkowski), e.g. the 8B8 antibody (ATCC HB 12070) or a humanized variant thereof.

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA*, 90:1746–1750 (1993); and Plowman et al., *Nature*, 366:473–475 (1993), including variants thereof such as the HER4 isoforms disclosed in WO 99/19488.

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2" "c-Erb-B2" and "HER2" when used herein refer to the human protein, and "erbB2," "c-erb-B2," and "her2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS (USA)* 82:6497–650 (1985)and Yamamoto et al. *Nature* 319:230–234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1–4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N-terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1–11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the "PI uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed-in the example herein; and nuclear/chromatin condensation-along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the

ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0–10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545–548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science*, 256:1205–1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.*, 14(3): 1909–1919 (1994); *Nature*, 362:312–318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177\text{-}244}$)

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.*, 269(20):14661–14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91–3242, Vol. I, pages 647–669 [1991]). The constant domains involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H–V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is use d in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal

antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., *Protein Eng.* 8(10): 1057–1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624–628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581–597 (1991), for example

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851–6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human inmmunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522–525 (1986); Reichmann et al., *Nature*, 332:323–329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593–596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269–315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H–V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444–6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see Example 1, below). Therapeutically effective amount also refers to a target serum concentration, such as a trough serum concentration, that has been shown to be effective in suppressing disease symptoms when maintained for a period of time.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY 117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (W B Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,

3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375–382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247–267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g.,an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

The term "serum concentration," "serum drug concentration," or "serum HERCEPTIN® anti-ErbB2 antibody concentration" refers to the concentration of a drug, such as HERCEPTIN® anti-ErbB2 antibody, in the blood serum of an animal or human patient being treated with the drug. Serum concentration of HERCEPTIN® anti-ErbB2 antibody, for example, is preferably determined by immunoassay. Preferably, the immunoassay is an ELISA according to the procedure disclosed herein.

The term "peak serum concentration" refers to the maximal serum drug concentration shortly after delivery of the drug into the animal or human patient, after the drug has been distributed throughout the blood system, but before significant tissue distribution, metabolism or excretion of drug by the body has occurred.

The term "trough serum concentration" refers to the serum drug concentration at a time after delivery of a previous dose and immediately prior to delivery of the next subsequent dose of drug in a series of doses. Generally, the trough serum concentration is a minimum sustained efficacious drug concentration in the series of drug administrations. Also, the trough serum concentration is frequently targeted as a minimum serum concentration for efficacy because it represents the serum concentration at which another dose of drug is to be administered as part of the treatment regimen. If the delivery of drug is by intravenous administration, the trough serum concentration is most preferably attained within 1 day of a front loading initial drug delivery. If the delivery of drug is by subcutaneous administration, the peak serum concentration is preferably attained in 3 days or less. According to the invention, the trough serum concentration is preferably attained in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, most preferably in 1 week or less, including 1 day or less using any of the drug delivery methods disclosed herein.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human patient over a period of time greater than approximately 5 minutes, preferably between approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human patient, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human patient,

preferably within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human patient, wherein the pump delivers a predetermined amount of drug for a predetermined period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human patient, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example,- by pinching or drawing the skin up and away from underlying tissue.

The term "front loading" when referring to drug administration is meant to describe an initially higher dose followed by the same or lower doses at intervals. The initial higher dose or doses are meant to more rapidly increase the animal or human patient's serum drug concentration to an efficacious target serum concentration. According to the present invention, front loading is achieved by an initial dose or doses delivered over three weeks or less that causes the animal's or patient's serum concentration to reach a target serum trough concentration. Preferably, the initial front loading dose or series of doses is administered in two weeks or less, more preferably in 1 week or less, including 1 day or less. Most preferably, where the initial dose is a single dose and is not followed by a subsequent maintenance dose for at least 1 week, the initial dose is administered in 1 day or less. Where the initial dose is a series of doses, each dose is separated by at least 3 hours, but not more than 3 weeks or less, preferably 2 weeks or less, more preferably 1 week or less, most preferably 1 day or less. To avoid adverse immune reaction to an antibody drug such as an anti-ErbB2 antibody (e.g., HERCEPTIN® anti-ErbB2 antibody) in an animal or patient who has not previously been treated with the antibody, it may be preferable to deliver initial doses of the antibody by intravenous infusion. The present invention includes front loading drug delivery of initial and maintenance doses by infusion or bolus administration, intravenously or subcutaneously.

Published information related to anti-ErbB2 antibodies includes the following issued patents and published applications: PCT/US89/00051,published Jan. 5,1989; PCT/US90/02697, published May 18,1990; EU 0474727 issued Jul. 23, 1997; DE 69031120.6, issued Jul. 23, 1997; PCT/US97/18385, published Oct. 9 1997; SA 97/9185, issued Oct. 14, 1997; U.S. Pat. No. 5,677,171, issued Oct. 14, 1997; U.S. Pat. No. 5,720,937, issued Feb. 24, 1998; U.S. Pat. No. 5,720,954, issued Feb. 24, 1998; U.S. Pat. No. 5,725,856, issued Mar. 10, 1998; U.S. Pat. No. 5,770,195, issued Jun. 23, 1998; U.S. Pat. No. 5,772,997, issued Jun. 30, 1998; PCT/US98/2626, published Dec. 10, 1998; and PCT/US99/06673, published Mar. 26, 1999, each of which patents and publications is herein incorporated by reference in its entirety.

II. Production of anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al., *PNAS (USA)* 88:8691–8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59–103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC- 11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51–63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells-is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59–103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-M EM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional imnmunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.*, 5:256–262 (1993) and Plückthun, *Immunol. Revs.*, 130:151–188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552–554 (1990). Clackson et al., *Nature*, 352:624–628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779–783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265–2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci. USA*, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import"variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522–525 (1986); Riechmann et al., *Nature*, 332:323–327 (1988); Verhoeyen et al., *Science* 239:1534–1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immnol.*, 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the

functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255–258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581–597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al. , *Journal of Biochemical and Biophysical Methods* 24:107–117(1992) and Brennan et al., *Science*, 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10: 163–167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537–539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target

immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217–225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547–1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444–6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies With the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM) :Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50–100%, the SKBR3 assay described in WO 89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish

(2 mls/35 mm dish) 2.5μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50–100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191–1195 (1992) and Shopes, B. *J. Immunol.* 148:2918–2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560–2565 (1993). Alternatively, an antibody can be engineered which has dual Fe regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219–230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as inununoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). S Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., *J. Biol. Chem.* 257: 286–288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al., *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO 81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active

portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature*, 312: 604–608 [1984]).

(xi) Antibody-salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H, region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163–167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1–13 [1983]). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5–4.5, preferably performed at low salt concentrations (e.g. from about 0–0.25M salt).

III. Determination of anti-ErbB2 Antibody Concentration in Serum

The following non-limiting assay is useful for determining the presence of and to quantitate the amount of specific rhuMAb HER2 (humanized anti-$p185^{HER2}$ monoclonal antibody, including HERCEPTIN® anti-ErbB2 antibody) in a body fluid of a mammal including, but not limited to, serum, amniotic fluid, milk, umbilical cord serum, ocular aqueous and vitreous liquids, and ocular vitreous gel.

Plate Binding Activity Assay for rhuMAb HER2 (Humanized Anti-$p185^{HER2}$ Monoclonal Antibody

The method of assaying rhuMAb HER2 described herein is meant as an example of such a method and is not meant to be limiting. A standardized preparation of rhuMAb HER2 (Genentech, Inc., South San Francisco, Calif.), controls, and serum samples were diluted with Assay Diluent (PBS/0.5% BSA/0.05% Polysorbate 20/0.01% Thimerosal). The dilutions of standardized rhuMAb HER2 were prepared to span a range of concentrations useful for a standard curve. The samples were diluted to fall within the standard curve.

An aliquot of Coat Antigen in Coating buffer (recombinant $p185^{HER2}$ (Genentech, Inc.) in 0.05 M sodium carbonate buffer) was added to each well of a microtiter plate and incubated at 2–8° C. for 12–72 hours. The coatin solution was removed and each well was washed six times with water, then blotted to remove excess water.

An aliquot of Assay Diluent was added to each well and incubated for 1–2 hours at ambient temperature with agitation. The wells were washed as in the previous step.

Aliquots of diluted standard, control and sample solutions were added to the wells and incubated at ambient temperature for 1 hour with agitation to allow binding of the antibody to the coating antigen. The wells are washed again with water as in previous steps.

Horse radish peroxidase-conjugate (HRP-conjugate, Goat anti-human IgG Fc conjugated to horseradish peroxidase; Organon Teknika catalog #55253 or equivalent) was diluted with Assay Diluent to yield an appropriate optical density range between the highest and lowest standards. An aliquot of the HRP-conjugate solution was added to each well and incubated at ambient temperature for 1 hour with agitation. The wells were washed with water as in previous steps.

An aliquot of Substrate Solution (o-phenylenediamine (OPD) 5 mg tablet (Sigma P6912 or equivalent) in 12.5 ml 4 mM H_2O_2 in PBS) was added to each well and incubated for a sufficient period of time (approximately 8–10 minutes) in the dark at ambient temperature to allow color development. The reaction was stopped with an aliquot of 4.5 N sulfuric acid. Optical density was read at 490–492 nm for detection absorbance and 405 nm for reference absorbance. The standard curve data are plotted and the results for the controls and samples are determined from the standard curve.

IV. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; met al complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial growth factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

V. Treatment With the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as

intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

The treatment of the present invention involves the administration of an anti-ErbB2 antibody to an animal or human patient, followed at intervals by subsequent doses of equal or smaller doses such that a target serum concentration is achieved and maintained during treatment. Preferably, maintenance doses are delivered by bolus delivery, preferably by subcutaneous bolus administration, making treatment convenient and cost-effective for the patient and health care professionals.

Where combined administration of a chemotherapeutic agent (other than an antracycline) is desired, the combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial growth factor (VEGF). Alternatively, or additionally, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. The ErbB2 antibody may be co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration, or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

In addition to the above therapeutic regimens, the patient may be subjected to surgical removal of cancer cells and/or or-radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of anti-ErbB2 antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Where the treatment involves a series of treatments, the initial dose or initial doses are followed at daily or weekly intervals by maintenance doses. Each maintenance dose provides the same or a smaller amount of antibody compared to the amount of antibody administered in the initial dose or doses.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1–20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy is easily monitored by conventional techniques and assays.

According to the invention, dosage regimens may include an initial dose of anti-ErbB2 of 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous infusion, followed by subsequent weekly maintenance doses of 2 mg/kg by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. Where the antibody is well-tolerated by the patient, the time of infusion may be reduced.

Alternatively, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

Another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

Still another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

As an alternative regimen, initial doses of 4 mg/kg anti-ErbB2 antibody may be administered on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

An additional regimen involves an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

Alternatively, the invention may include a cycle of dosing in which delivery of anti-ErbB2 antibody is 2–3 times per week for 3 weeks. The 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The invention further includes a cyclic dosage regimen in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms. Further information about suitable dosages is provided in the Examples below.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture may comprise a package inserts with instruc-

tions for use, including, e.g., a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin or epirubicin.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLES

Example 1

Preparation and Efficacy of HERCEPTIN® Anti-ErbB2 Antibody

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 $IgG_1\kappa$ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50: 1550–1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2–3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al., *Proc. Natl. Acad. Sci.* (*USA*) 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN® anti-ErbB2 antibody). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285–4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for $p185^{HER2}$ (Dillohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of p_{185}^{HER2}, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® anti-ErbB2 antibody is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria

Patients had to fulfill all of the following criteria to be eligible for study admission:

Metastatic breast cancer

Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffim-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for p_{185}^{HER2}].

Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

The ability to understand and willingness to sign a written informed consent form

Women ≧18 years

Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria

Patients with any of the following were excluded from study entry:

Prior cytotoxic chemotherapy for metastatic breast cancer

Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.

Concomitant malignancy that has not been curatively treated

A performance status of <60% on the Karnofsky scale

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® anti-ErbB2 antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered

intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regimens for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® anti-ErbB2 antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3–5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H_2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease

Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response

Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response

A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response

A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease

No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed.2), New York, N.Y., Wiley, 1981, pp 13–17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN® anti-ErbB2 antibody, without increase in overall severe adverse events (AE):

TABLE 1

HERCEPTIN ® Anti-ErbB2 Antibody Efficacy

	Enrolled	TTP (months)	RR (%)	AE (%)
CRx	234	5.5	36.2	66
CRx + 14	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*$p < 0.001$ by log-rank test; ** $p < 0.01$ by X^2 test; CRx: chemotherapy; AC: anthracycline/cyclophosphamide treatment; H: HERCEPTIN ® anti-ErbB2 antibody; T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade ¾) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor treatment with HERCEPTIN® anti-ErbB2 antibody and paclitaxel (TAXOL®) where a combined treatment regimen is desired.

Example 2

Pharmacokinetic and Pharmacodynamic Properties of Anti-ErbB2 Antibody (HERCEPTIN®)

HERCEPTIN® anti-ErbB2 antibody was administered by intravenous, infusion to human patients selected according to the criteria provided in Example 1. An initial dose of 4 mg/kg HERCEPTIN® anti-ErbB2 antibody was delivered by intravenous infusion, followed by subsequent i.v. infusions of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody weekly for several weeks. Two hundred thirteen patients began this treatment regimen and serum drug concentration was obtained beyond 8 weeks for fewer than 90 patients as selective discontinuation of patients with rapidly progressing disease occurred. Of the 213 patients who began treatment, serum trough concentration data were available for 80 patients at Week 12, for 77 patients at Week 16, for 44 patients at Week 20, for 51 patients at Week 24, for 25 patients at Week 28, for 23 patients at Week 32, and for 37 patients at Week 36.

HERCEPTIN® anti-ErbB2 Antibody Trough Serum Concentrations for Weeks 0–36

The HERCEPTIN® anti-ErbB2 antibody trough serum concentrations (μg/ml, mean±SE) from Week 2 through Week 36 are plotted in FIG. **3** (dark circles). The number of patients was fairly constant because data from patients discontinued from the program due to rapidly progressing disease were excluded from this analysis. Trough serum concentrations tended to increase through Week 12 and tended to plateau after that time.

HERCEPTIN® anti-ErbB2 Antibody Trough and Peak Serum Concentrations for Weeks 1–8

Some HERCEPTIN® anti-ErbB2 antibody serum concentration data were available for 212 of the original 213 patients. Trough and peak serum concentration data reflecting the first HERCEPTIN® anti-ErbB2 antibody infusion

were available for 195 of the 212 patients. For the seventh infusion, trough serum concentration data were available for 137/212 patients and peak serum concentration data were available for 114/212 patients. Table 2 presents a summary of statistics from trough and peak serum concentrations for the first 8 weeks of treatment. Peak samples were drawn shortly after the end of HERCEPTIN® anti-ErbB2 antibody administration; trough samples were drawn prior to the subsequent dose (i.e., 1 week later). Serum concentrations of HERCEPTIN® anti-ErbB2 antibody were determined as disclosed herein.

TABLE 2

HERCEPTIN ® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for the First 8 Weeks of Treatment (μg/ml)

	Dose Number	n	Mean	SD	Minimum	Maximum
Peak	1	195	100.3	35.2	30.7	274.6
Trough		195	25.0	12.7	0.16	60.7
Peak	2	190	74.3	31.3	20.8	307.9
Trough		167	30.4	16.0	0.2	74.4
Peak	3	167	75.3	26.8	16.1	194.8
Trough		179	33.7	17.9	0.2	98.2
Peak	4	175	80.2	26.9	22.2	167
Trough		132	38.6	20.1	0.2	89.4
Peak	5	128	85.9	29.2	27.8	185.8
Trough		141	42.1	24.8	0.2	148.7
Peak	6	137	87.2	32.2	28.9	218.1
Trough		115	43.2	24.0	0.2	109.9
Peak	7	114	89.7	32.5	16.3	187.8
Trough		137	48.8	24.9	0.2	105.2
Peak	8	133	95.6	35.9	11.4	295.6

The data in Table 2 suggest that there was an increase in trough serum concentration over time. Of the many patients studied, there were 18 patients for whom the trough concentrations did not exceed 20 μg/ml from Week 2 through Week 8. A HERCEPTIN® anti-ErbB2 antibody trough serum concentration of 20 μg/ml was nominally targeted for these studies based on prior pharmacologic studies in animals and exploratory analyses in clinical trials.

Patient response status was evaluated relative to serum concentration of HERCEPTIN® anti-ErbB2 antibody. For this purpose, mean serum concentration (an average of troughs and peaks) was calculated for various times and patient response status (where the patient response status was determined by an independent Response Evaluation Committee). The increase in serum concentration between Weeks 2 and 8 appeared to be greater in responders than in nonresponders, suggesting that there is a relationship between response status and HERCEPTIN® anti-ErbB2 antibody serum concentration. A statistical analysis (analysis of variance) of trough serum concentration values at Week 2 and an average of Weeks 7 and 8 in relation to response status indicated a highly significant relationship between response status and average trough of Weeks 7 and 8 ($p<0.001$). The results indicated that there was a significant difference between the trough serum concentration (average troughs of Weeks 7 and 8) in the responders and nonresponders: trough concentrations were 60±20 μg/ml in the responders versus 44±25 μg/ml in the nonresponders (mean±SD). HER2 overexpression level and type of metastatic sites were associated with significant differences in trough serum concentrations. At Week 2, patients with 2+ HER2 overexpression had significantly higher trough serum concentrations (n=40, mean=28.8 μg/ml, SD=10.4) compared with patients with 3+ HER2 overexpression (n=155, mean=24.1 μg/ml, SD=13.1). This difference in the average trough serum concentrations for Weeks 7 and 8 was no longer statistically significant. Further, at Week 2, patients with superficial disease had significantly higher trough serum concentrations (n=12, mean 34.1 μg/ml, SD=12.0) compared with patients with visceral disease (n=183, mean=24.4 μg/ml, SD=12.6). This difference in the average trough serum concentrations for Weeks 7 and 8 was significant. These data indicate that the rise in trough serum concentrations between Weeks 2 and 7/8 occurs for human patients with various disease profiles.

In a subsequent, similarly designed study, human breast cancer patients were treated with a loading dose of 8 mg/kg followed by maintenance doses of 4 mg/kg weekly. The results of this preliminary human study indicated that an 8mg/kg load:4 mg/kg weekly maintenance regimen was efficacious in reducing tumor volume in the patients.

The data disclosed in this Example indicate that front loading of antibody, such that a target serum concentration is reached more quickly, may be associated with improved outcomes.

Example 3

I.V. Bolus Delivery and Subcutaneous Infusion of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

The efficacy of infusion or bolus delivery of humanized anti-ErbB2 antibody (HERCEPTIN®, see Example 1 for preparation), either by intravenous injection or subcutaneous injection, was examined. The purpose of the study was to ask whether subcutaneous delivery was feasible and whether the convenient subcutaneous bolus delivery was useful in treating metastatic breast cancer in animals inoculated with a cell line that overexpresses the HER2 gene. The results, detailed below, show that i.v. and s.c. infusion and bolus delivery are feasible treatment methodologies.

A study in a nude mouse xenograft model, which incorporates a human breast cancer cell line that naturally overexpresses the HER2 gene (BT-474MI, derived from BT-474 cells, ATCC Accession number HTB-20), comparing tumor volume as a function of i.v. bolus versus s.c. infusion was performed as follows. In the first study athymic nude nu nu 7–9 week old female mice were obtained from Taconic Inc (Germantown, N.Y.). To initiate tumor development, each mouse was inoculated subcutaneously with 3×10^6 BT474M 1 cells suspended in Matrigel™. When tumor nodules reached a volume of approximately 100 mm^3, animals were randomized to 4 treatment groups. The groups were treated according to Table 3.

TABLE 3

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Infusion

Group, Dose, Antibody	Target Serum Conc. μg/ml	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose
1-Control, rhuMAb E25	20	IV LD and SC infusion	2.20	0.250 mg/ml (infusate)
2-Low Dose SC rhuMAb HER2	1	IV LD and SC infusion	0.313	0.050 mg/ml (infusate)
3-High Dose SC rhuMAb HER2	20	IV LD and SC infusion	6.25	1.00 mg/ml (infusate)
4-IV Multi-Dose rhuMAb HER2	20 (trough)	IV LD and MD	4.00	2 mg/kg/week (IV bolus)

Serum Conc. = concentration in serum.
LD = loading dose.
MD = maintenance dose.
Infusate concentration was calculated to achieve targeted serum concentration using Alzet ® osmotic minipumps (Alza Corp., Palo Alto, CA).

Animals were exposed to estrogen by subcutaneous sustained release estrogen pellet 9 days before the start of

dosing to promote growth of grafted tumor cells. The animals were inoculated with the BT474M 1 cells 8 days before the beginning of treatment and tumors were allowed to grow. The animals were then treated with nonrelevant antibody E25 (non-specific for HER2 receptor, but a member of the monoclonal IgG class) or test antibody HERCEPTIN® anti-ErbB2 anitbody as indicated in Table 3. The dosage levels were selected to achieve target serum concentrations of HERCEPTIN®, either 1 μg/ml or 20 μg/ml, by subcutaneous pump infusion or by i.v. bolus delivery. The study groups were treated until day 35. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly (Oust prior to dosing for Group 4) using 3 mice/group/time point. The anti-ErbB2 antibody concentration was determined according to the method disclosed herein involving standard techniques. Tumor volumes were measured two days before dosing began and twice per week from day 6 to day 35 in the study for which data is tabulated below. Tumors were measured in three dimensions and volumes were expressed in mm^3. Efficacy was determined by a statistical comparison (ANOVA) of tumor volumes of test animals relative to untreated control animals.

As shown in Table 4, below, treatment of the BT474M 1 tumor-bearing mice with HERCEPTIN® anti-ErbB2 antibody by the indicated dosage methods significantly inhibited the growth of the tumors. All HERCEPTIN® -treated groups showed similar inhibition of tumor growth relative to the control group. No dose-response was observed.

TABLE 4

Comparison of S.C. Infusion and I.V. Bolus Delivery

Treatment Group	Tumor Volume (mm^3), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6–Day 35 (n = 13)	HERCEPT ® Serum Conc. (μg/ml), Day 27, (n = 3)
control s.c. infusion	764 ± 700	5650 ± 4700	4.16 ± 1.94
s.c. infusion (low dose)	80.6 ± 158	1610 ± 1250	2.11 ± 1.74
s.c. infusion (high dose)	31 ± 75.6	1440 ± 1140	22.1 ± 5.43
i.v. bolus dose*	49.7 ± 95.7	2150 ± 1480	21.7 ± 17.1**

s.c. = subcutaneous delivery; i.v. = intravenous delivery.
*4.0 mg/kg Loading Dose and 2.0 mg/kg/week Maintenance Dose.
**at predose (trough serum concentration immediately prior to a maintenance dose)

The results tabulated above indicate that maintenance of a serum concentration of approximately 2 μg/ml was as effective as a concentration of 20 μg/ml in this study. The results indicated that dosing by subcutaneous infusion was as effective as intravenous bolus dosing and achieved similar trough serum concentrations. The results also indicate that the dose levels studied are at the top of the dose-response curve in this model and that subcutaneous dosing is effective in treating breast cancer tumors. Thus, subcutaneous administration of maintenance doses is feasible as part of a HERCEPTIN® anti-ErbB2 antibody treatment regimen.

Example 4

I.V. Bolus and Subcutaneous Bolus Deliveries of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

Subcutaneous bolus delivery is convenient and cost-effective for the patient and health care professionals. The results of the study disclosed in this example indicate that subcutaneous bolus delivery was as effective as intravenous bolus delivery in reducing breast cell tumor size in a mouse.

This study was set up as disclosed herein in Example 3 for the comparison of intravenous bolus and subcutaneous infusion delivery. A sustained release estrogen implant was inserted subcutaneously one day before tumor cell innoculation as described in Example 3. Six days after tumor cell innoculation, the initial tumor measurement was performed. Seven days after tumor cell innoculation, the first dose of control antibody or HERCEPTIN® anti-ErbB2 antibody was delivered. The animal groups, type of delivery, loading dose and maintenance doses are provided in Table 4. Animals were dosed once weekly for 4 weeks.

TABLE 5

Animal Groups and Doses for Comparison of I.V. Bolus and S.C Bolus Delivery

Group	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose (mg/kg/week)	n
1-Control rbuMAb E25	IV	8	4	10
2-rhuMAb HER2	IV	2	1	10
3-rhuMAb HER2	IV	4	2	10
4-rhuMAb HER2	IV	8	4	10
5-rhuMAb HER2	SC	4	2	10

IV = intraveneous; SC = subcutaneous; n = number of animals per group.

The mice were treated according to the information in Table 4 and using the techniques disclosed in Example 3. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly before each weekly i.v. maintenance dose according to the procedure described herein and using standard techniques. The control E25 antibody serum concentration was determined according to standard immunoassay techniques. Table 6 shows the increase in HERCEPTIN® anti-ErbB2 antibody serum concentrations with time.

TABLE 6

IV versus SC Bolus Delivery:
Serum HERCEPTIN ® Anti-ErbB2 Antibody Concentration
Serum Concentration, μg/ml

Treatment Group (delivery, MD)	Day 0 Mean (SD)	Day 7 Mean (SD)	Day 14 Mean (SD)	Day 21 Mean (SD)
1-Control rhu MAb E25	0	25.9	34.6	38.5
(IV, 4mg/kg)	(0)	(8.29)	(11.2)	(14.4)
2-rhu MAb HER2	0	4.96	8.55	8.05
(IV, 1 mg/kg)	(0)	(3.79)	(5.83)	(4.67)
3-rhu MAb HER2	0	13.4	18.9	22.6
(IV, 2 mg/kg)	(0)	(9.24)	(12.0)	(9.21)
4-rhu MAb HER2	0	29.6	37.7	46.2
(IV, 4 mg/kg)	(0)	(13.5)	(14.4)	(13.8)
5-rhu MAb HER2	0	12.5	16.9	17.6
(SC, 2 mg/kg)	(0)	(7.33)	(10.2)	(10.7)

n = 10 for time points Days 0, 7 and 14.
N = 9 for Day 21.

Table 7 shows the relative efficacy of intravenous bolus delivery and subcutaneous bolus delivery for Groups 1–5 having achieved the serum antibody concentrations presented in Table 6. For this study, efficacy was measured as a decrease in tumor volume. Tumor volume was measured twice weekly.

TABLE 7

Efficacy of HERCEPTIN ® Anti-ErbB2 Antibody Measured as a Change in Tumor Volume Comparing Intravenous Bolus and Subcutaneous Bolus Delivery, Mean (SD)

Treatment Group (Delivery, MD)	Tumor Vol. Day 6, mm^3	Tumor Vol. Day 28 mm^3	Tumor Vol. Day 31, mm^3	Day 6–Day 31* Area Under Curve Tumor Vol., mm^3	Tumor Growth Rate on Log (TM + 1)
1-IV Control	321	1530	1630	13600	0.0660
	(190)	(1040)	(1170)	(7230)	(0.0200)
2-IV Herceptin	297	175	151	4690	–0.0505
1 mg/kg	(130)	(215)	(188)	(1400)	(0.142)
3-IV Herceptin	269	75.7	73.6	3510	–0.0608
2 mg/kg	(129)	(92.4)	(84.5)	(1220)	(0.110)
4-IV Herceptin	272	25.3	25.8	2880	–0.0810
4 mg/kg	(117)	(75.9)	(72.9)	(1230)	(0.0859)
5-SC Herceptin	268	76.2	90.4	3230	–0.0304
2 mg/kg	(117)	(98.8)	(105)	(1440)	(0.104)

N = 10 for each data point.
TM = tumor measurement.
IV = intravenous.
SC = subcutaneous.
MD = maintenance dose.
Tumor Vol. = tumor volume, mm^3.
*Day 17 excluded due to measurement error.
Tumor growth rate calculated on Day 21–Day 31 Log(TM + 1). Area under the curve is the area beneath a plot of tumor volume versus time.

FIGS. **4**A and **4**B are graphical plots of changes in tumor volume over time, some of which data is found in Table 7. FIG. **4**A is a linear plot of tumor volume versus time. FIG. **4**B is a semilogarithmic plot of the same data, allowing the test points be viewed more clearly. The data in Table 7 and FIGS. **4**A and **4**B indicate that, although a dose-related response was not observed between HERCEPTIN-treated groups, dosing by subcutaneous bolus was as effective as intravenous bolus dosing and achieved similar trough serum concentrations.

Example 5

Regimens for Intravenous and Subcutaneous Delivery of Anti-ErbB2 Antibody

According to the invention, methods of anti-ErbB2 antibody (e.g., HERCEPTIN®) delivery comprise greater front loading of the drug to achieve a target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less. According to the invention, this initial dosing is followed by dosing that maintains the target serum concentration by subsequent doses of equal or smaller amount. An advantage of the methods of the invention is that the maintenance dosing may be less frequent and/or delivered by subcutaneous injection, making the treatment regimens of the invention convenient and cost-effective for the patient and medical professionals administering the antibody. In addition, a subcutaneous maintenance dose regimen may be interrupted by intravenous dosing (such as infusion) when the patient's chemotherapy requires delivery of other drugs by intravenous injection.

To test the following dosage regimens, human subjects are selected according to the criteria disclosed in Example 1, above. The number of initial doses is one or more doses sufficient to achieve an efficacious target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The number of maintenance doses may be one or more doses sufficient to achieve suppression of disease symptoms, such as a decrease in tumor volume. The maintenance doses are equal to or smaller than the initial dose or doses, consistent with an object of the invention of administering HERCEPTIN® anti-ErbB2 antibody by regimens providing greater front loading. The specific drug delivery regimens disclosed herein are representative of the invention and are not meant to be limiting.

In one trial, an initial dose of 6 mg/kg, 8 mg/kg, or 12 mg/kg of HERCEPTIN® anti-ErbB2 antibody is delivered to human patients by intravenous or subcutaneous injection. Initial doses (loading doses) are delivered by intravenous infusion or bolus injection or preferably subcutaneous bolus injection. Preferably a target trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10–20 μg/ml is achieved.(averaged for all patients in the treatment group) and maintained by subsequent doses of anti-ErbB2 antibody that are equal to or smaller than the initial dose. In one method, a target trough serum concentration is achieved and maintained by once-per-week deliveries of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody by intravenous or subcutaneous injection for at least eight weeks. Alternatively, for this or any dosage regimen disclosed herein, subcutaneous continuous infusion by subcutaneous pump is used to delivery subsequent maintenance doses.

In another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10–20 μg/ml, averaged for an entire treatment group.

In another method, an initial (front loading) dose of 12 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10–20 μg/ml.

In yet another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous infusion or bolus injection, or preferably by subcutaneous bolus injection or infusion.

This is followed by administration of 8 mg/kg per week or 8 mg/kg per 2–3 weeks to maintain a trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10–20 μg/ml. Maintenance doses are delivered by intravenous infusion or bolus injection, or preferably by subcutaneous infusion or bolus injection.

In another method, the front loading initial dose is a series of intravenous or subcutaneous injections, for example, one on each of days 1, 2, and 3 of at least 1 mg/kg for each injection (where the amount of anti-ErbB2 antibody delivered by the sum of initial injections is more than 4 mg/kg), followed by maintenance doses of 6 mg/kg once each 3 week interval to maintain a target trough serum concentration (for example, approximately 10–20 μg/ml) of HERCEPTIN® anti-ErbB2 antibody. The maintenance doses are delivered by intravenous infusion or bolus injection or by subcutaneous infusion or subcutaneous bolus injection.

In yet another method, the front loading is by intravenous infusion of at least 1 mg/kg, preferably 4 mg/kg on each of five consecutive days, followed by repeats of this cycle a sufficient number of times to achieve suppression of disease symptoms. Following the initial dose or doses, subsequent doses may be delivered by subcutaneous infusion or bolus injection if tolerated by the patient. Such subcutaneous delivery is convenient and cost-effective for the patient and administering health care professionals.

In still another method, HERCEPTIN® anti-ErbB2 antibody is delivered initially as at least 2 intravenous infusions per week for three weeks, followed by repeats of this cycle to maintain an efficacious trough serum concentration of HERCEPTIN® 0 anti-ErbB2 antibody. The dose is at least 4 mg/kg of anti-ErbB2 antibody, preferably at least 5 mg/kg. The maintenance drug deliveries may be intravenous or subcutaneous.

Where the animal or patient tolerates the antibody during and after an initial dose, delivery of subsequent doses may be subcutaneous, thereby providing greater convenience and cost-effectiveness for the patient and health care professionals.

In animal studies, an initial dose of more than 4 mg/kg, preferably more than 5 mg/kg delivered by intravenous or subcutaneous injection, is followed by subcutaneous bolus injections of 2 mg/kg twice per week (separated by 3 days) to maintain a trough serum concentration of approximately 10–20 μg/ml. In addition, where the animal or patient is known to tolerate the antibody, an initial dose of HERCEPTIN® anti-ErbB2 antibody is optionally and preferably deliverable by subcutaneous bolus injection followed by subcutaneous maintenance injections.

While target serum concentrations are disclosed herein for the purpose of comparing animal studies and human trials, target serum concentrations in clinical uses may differ. The disclosure provided herein guides the user in selecting a front loading drug delivery regimen that provides an efficacious target trough serum concentration.

The methods of the invention disclosed herein optionally include the delivery of HERCEPTIN® anti-ErbB2 antibody in combination with a chemotherapeutic agent (other than an anthrocycline derivative) to achieve suppression of disease symptoms. The chemotherapeutic agent may be delivered with HERCEPTIN® anti-ErbB2 antibody or separately and according to a different dosing schedule. For example, subcutaneous delivery of HERCEPTIN® anti-ErbB2 antibody with TAXOL® is included in the invention. In addition, intravenous or subcutaneous injection of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody, followed by intravenous or subcutaneous injection of 6 mg/kg HERCEPTIN® anti-ErbB2 antibody every 3 weeks is administered in combination with a chemotherapeutic agent, such as a taxoid (e.g. paclitaxel 175mg/m^2 every 3 weeks) or an anthracycline derivative (e.g. doxorubicin 60 mg/m^2 or epirubicin 75 mg/m^2 every 3 weeks). Optionally, where an anthracycline derivative is administered, a cardioprotectant (e.g. 600 mg/m^2 cyclophosphamide every 3 weeks) is also administered. In another combination therapy, anti-ErbB2 antibody is administered in a loading dose of more than 4 mg/kg, preferably more than 5 mg/kg, and more preferably at least 8 mg/kg. The loading dose is followed by maintenance doses of at least 2 mg/kg weekly, preferably 6 mg/kg every 3 weeks. The combination therapy includes administration of a taxoid during treatment with anti-ErbB2 antibody. According to one embodiment of the invention, the taxoid is paclitaxel and is administered at a dose of 70–100 mg/m^2/week. According to another embodiment of the invention, the taxoid is docetaxel and is administered at a dose of 30–70 mg/m^2/week.

Example 6

HERCEPTIN® Administered Intravenously Every Three Weeks in Combination with Paclitaxel

Currently, the recommended dose of HERCEPTIN® is 2 mg/kg once weekly. Patients will be administered HERCEPTIN® every three weeks instead of weekly, along with paclitaxel (175 mg/m^2 every three weeks). Simulation of the proposed treatment regimen suggests that the trough serum concentrations will be 17 mcg/ml, in the range (10–20mcg/ml) of the targeted trough serum concentrations from previous HERCEPTIN® IV clinical trials. After the first 12 patients the PK parameters will be assessed, if exposure is felt inadequate, then the dose will be increased to 8 mg/kg every three weeks for the remaining 12 patients.

Inclusion Criteria

1) Females≧18 years of age
2) Histologically confirmed ErbB2 over-expressing metastatic breast cancer
3) Patients who have been newly diagnosed with metastatic disease
4) Have a Karnofsky performance status of≧70%
5) Give written informed consent prior to any study specific screening procedures with the understanding that the patient has the right to withdraw from the study at any time, without prejudice.

Exclusion Criteria

1) Pregnant or lactating women
2) Women of childbearing potential unless (1) surgically sterile or (2) using adequate measures of contraception such as oral contraceptive, intra-uterine device or barrier method of contraception in conjunction with spermicidal jelly.
3) Clinical or radiologic evidence of CNS metastases.
4) History of any significant cardiac disease
5) LVEF≦50%
6) No prior taxane therapy in any treatment setting.
7) Any of the following abnormal baseline hematologic values:
   Hb less than 9 g/dl
   WBC less than 3.0×10^9/l
   Granulocytes less than 1.5×109/l
   Platelets less than 100×109/l
8) Any of the following abnormal baseline liver function tests:
   Serum bilirubin greater than 1.5× ULN (upper normal limit)
   ALT and/or AST greater than 2.5× ULN (greater than 4.0× ULN if liver or bone metastasis)
   Alkaline phosphatase greater than 2.5× ULN (greater than 4.0× ULN if liver or bone metastasis)
9) The following abnormal baseline renal function tests: serum creatinine greater than 1.5× ULN
10) History of other serious medical conditions that would preclude patient participation in an investigational study.

HERCEPTIN® Loading dose and schedule: 8 mg/kg for first dose. Maintenance dose and schedule: 6 mg/kg every 3 weeks.

Paclitaxel—175 mg/m^2 IV every 3 weeks×6 cycles as a 3-hour infusion. NOTE: On the first cycle of treatment,

paclitaxel will be dosed 8 hours prior to HERCEPTIN® to determine the PK of paclitaxel alone. HERCEPTIN® will be administered 8 hours post-paclitaxel for the 1st cycle only. In subsequent treatment cycles, HERCEPTIN® will be administered prior to paclitaxel.

The total duration of this study is 18 weeks. Study subjects will receive up to 6 total HERCEPTIN® doses. After the last subject has received the last cycle of paclitaxel, data collection for safety and pharmacokinetic analysis will stop, and the study will close to protocol specified treatment. Study subjects may continue to receive the HERCEPTIN® +/− paclitaxel at the discretion of the investigator.

It is believed that the above treatment regimen will be effective in treating metastatic breast cancer, despite the infrequency with which HERCEPTIN® is administered to the patient.

While the particular aspects and embodiments of the invention as herein shown and disclosed in detail is fully capable of obtaining the objects and providing the advantages herein before stated, it is to be understood that it is merely illustrative of some of the presently preferred embodiments of the invention and that no limitations are intended to the details of methods and articles of manufacture shown other than as described in the appended claims. The disclosures of all citations in the specification are expressly incorporated herein by reference.

SEQUENCE LISTING

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Val Gln Gly Asn Leu Glu Leu Thr Tyr Leu Pro Thr Asn Ala Ser
             35                  40                  45

Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu
             50                  55                  60

Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg
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Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
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Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
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Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
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Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
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Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
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Ile Gly Val Ala Trp Tyr Gln Gln Arg Pro Gly Gln Ser Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Asp
                 50                  55                  60

Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
                 65                  70                  75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
                 80                  85                  90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu
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Ile Lys
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Asp Tyr Thr Met Asp Trp Val Lys Gln Ser His Gly Lys Ser Leu
                 35                  40                  45
```

-continued

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Glu Trp Ile Gly Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                 50                  55                  60

Asn Gln Arg Phe Lys Gly Lys Ala Ser Leu Thr Val Asp Arg Ser
                 65                  70                  75

Ser Arg Ile Val Tyr Met Glu Leu Arg Ser Leu Thr Phe Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                 95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
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Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 100                 105

Ile Lys


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Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
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Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                 50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
                 65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                 95                 100                 105
```

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Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
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Ile Lys


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Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
                95                 100                 105

Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115
```

What is claimed is:

1. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising:

administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB2 antibody; and

administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose, wherein

the subsequent doses are separated in time from each other by at least two weeks.

2. The method of claim **1**, wherein the initial dose is at least approximately 6 mg/kg.

3. The method of claim **2**, wherein the initial dose is at least approximately 8 mg/kg.

4. The method of claim **3**, wherein the initial dose is at least approximately 12 mg/kg.

5. The method of claim **1**, wherein the subsequent doses are separated in time from each other by at least three weeks.

6. The method of claim **1**, wherein the initial dose is administered by intravenous injection, and wherein at least one subsequent dose is administered by subcutaneous injection.

7. The method of claim **1**, wherein the initial dose is administered by intravenous injection, wherein at least two subsequent doses are administered, and wherein each subsequent dose is administered by a method selected from the group consisting of intravenous injection and subcutaneous injection.

8. The method of claim **1**, wherein the initial dose and at least one subsequent dose are administered by subcutaneous injection.

9. The method of claim **1**, wherein the initial dose is selected from the group consisting of approximately 6 mg/kg, 8 mg/kg, or 12 mg/kg, wherein the plurality of subsequent doses are at least approximately 2 mg/kg.

10. The method of claim **9**, wherein the plurality of subsequent doses are separated in time from each other by at least three weeks.

11. The method of claim **10**, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

12. The method of claim **10**, wherein the initial dose is approximately 12 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

13. The method of claim **9**, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 8 mg/kg.

14. The method of claim **9**, wherein the initial dose is approximately 8 mg/kg, wherein at least one subsequent dose is 8 mg/kg, and wherein administration of the initial dose and subsequent doses are separated in time by at least 2 weeks.

15. The method of claim **14**, wherein the initial dose and subsequent doses are separated in time by at least 3 weeks.

16. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising:

administering to the patient an initial dose of the antibody, wherein the initial dose is a plurality of doses, wherein each of the plurality of initial doses is at least approximately 1 mg/kg and is administered on at least 3 consecutive days, and administering to the patient at least 1 subsequent dose of the antibody, wherein at least one subsequent dose is at least approximately 6 mg/kg, and wherein administration of the last initial dose and the first subsequent and additional subsequent doses are separated in time by at least 3 weeks.

17. The method of claim **1**, wherein said cancer is selected from the group consisting of breast cancer, leukemia, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

18. The method of claim **17**, wherein said cancer is breast cancer.

19. The method of claim **18**, wherein said cancer is metastatic breast carcinoma.

20. The method of claim **1**, wherein said antibody binds to the extracellular domain of the ErbB2 receptor.

21. The method of claim **20**, wherein said antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

22. The method of claim **21**, wherein said antibody is a humanized 4D5 anti-ErbB2 antibody.

23. The method of claim **1**, wherein efficacy is measured by determining the time to disease progression or the response rate.

24. A method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by two or more subsequent doses of the antibody, wherein the subsequent doses are separated in time from each other by at least two weeks.

25. The method of claim **24**, wherein the first dose and a first subsequent dose are separated from each other in time by at least about three weeks.

26. The method of claim **24**, wherein the first dose and subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

27. The method of claim **26**, wherein the first dose and subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

28. The method of claim **27**, wherein the first dose and subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

29. The method of claim **24**, wherein from about two to about ten subsequent doses of the antibody are administered to the patient.

30. The method of claim **24**, wherein the subsequent doses are separated in time from each other by at least about three weeks.

31. The method of claim **24**, wherein the two or more subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

32. The method of claim **24**, wherein the two or more subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

33. The method of claim **24**, wherein the two or more subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

* * * * *

EXHIBIT H

US007371379B2

(12) **United States Patent**
Baughman et al.

(10) **Patent No.: US 7,371,379 B2**
(45) **Date of Patent: May 13, 2008**

(54) **DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES**

(75) Inventors: **Sharon A. Baughman**, Ventura, CA (US); **Steven Shak**, Burlingame, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 540 days.

(21) Appl. No.: **10/600,152**

(22) Filed: **Jun. 20, 2003**

(65) **Prior Publication Data**

US 2004/0037824 A1 Feb. 26, 2004

Related U.S. Application Data

(62) Division of application No. 09/648,067, filed on Aug. 25, 2000, now Pat. No. 6,627,196.

(60) Provisional application No. 60/213,822, filed on Jun. 23, 2000, provisional application No. 60/151,018, filed on Aug. 27, 1999.

(51) **Int. Cl.**
A61K 39/395 (2006.01)

(52) **U.S. Cl.** **424/138.1**; 424/130.1; 424/133.1; 424/141.1; 424/142.1; 424/143.1; 424/155.1; 424/156.1; 424/174.1

(58) **Field of Classification Search** 424/130.1, 424/133.1, 138.1, 141.1, 142.1, 143.1, 155.1, 424/156.1, 174.1
See application file for complete search history.

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(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with anti-ErbB2 antibody.

40 Claims, 5 Drawing Sheets

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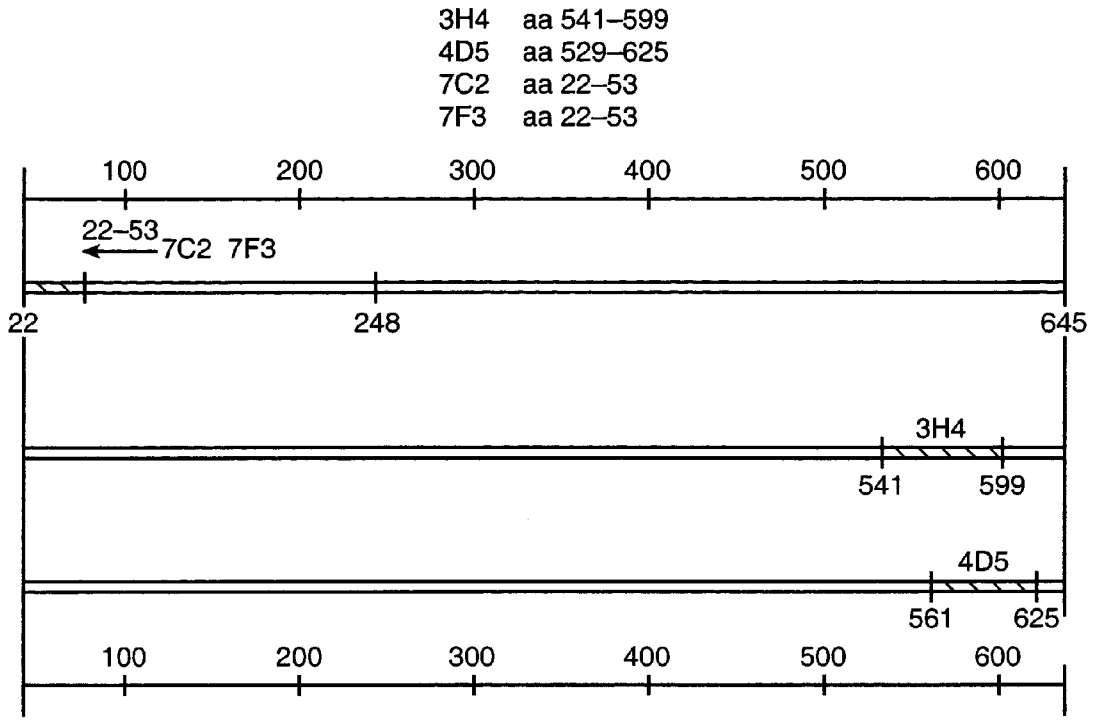

3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR
541 **599**

4D5 epitope (SEQ ID NO:9) 64 residues

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP
561 **625**

FIG._1

```
1    MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPA
38   SPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFL
75   QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN
112  YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149  LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186  TNRSRA
```

FIG._2

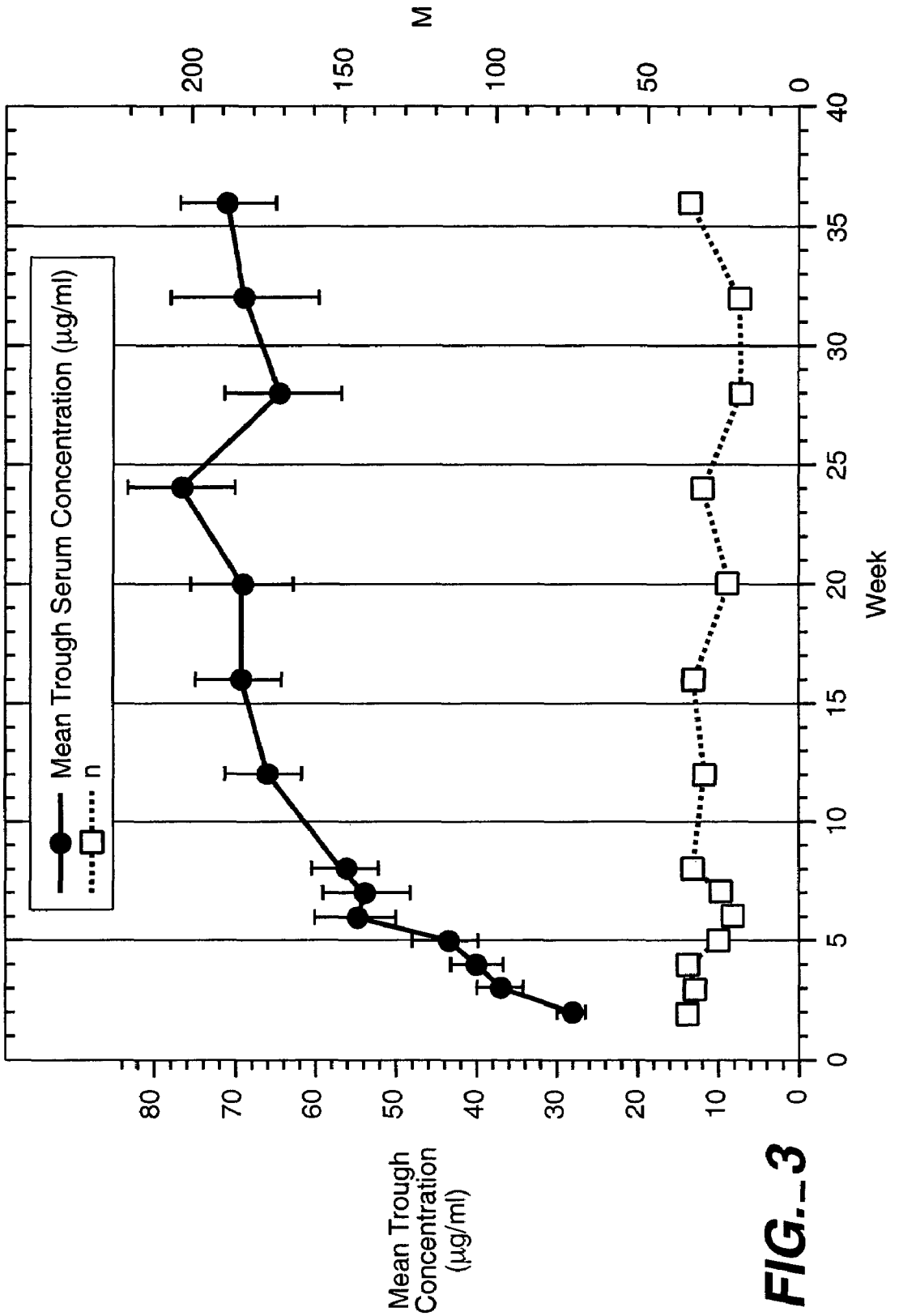
Mean Trough Serum Concentration (µg/ml)
n
Mean Trough Concentration (µg/ml)
0
10
20
30
40
50
60
70
80
n
0
50
100
150
200
Week
0
5
10
15
20
25
30
35
40

FIG._3

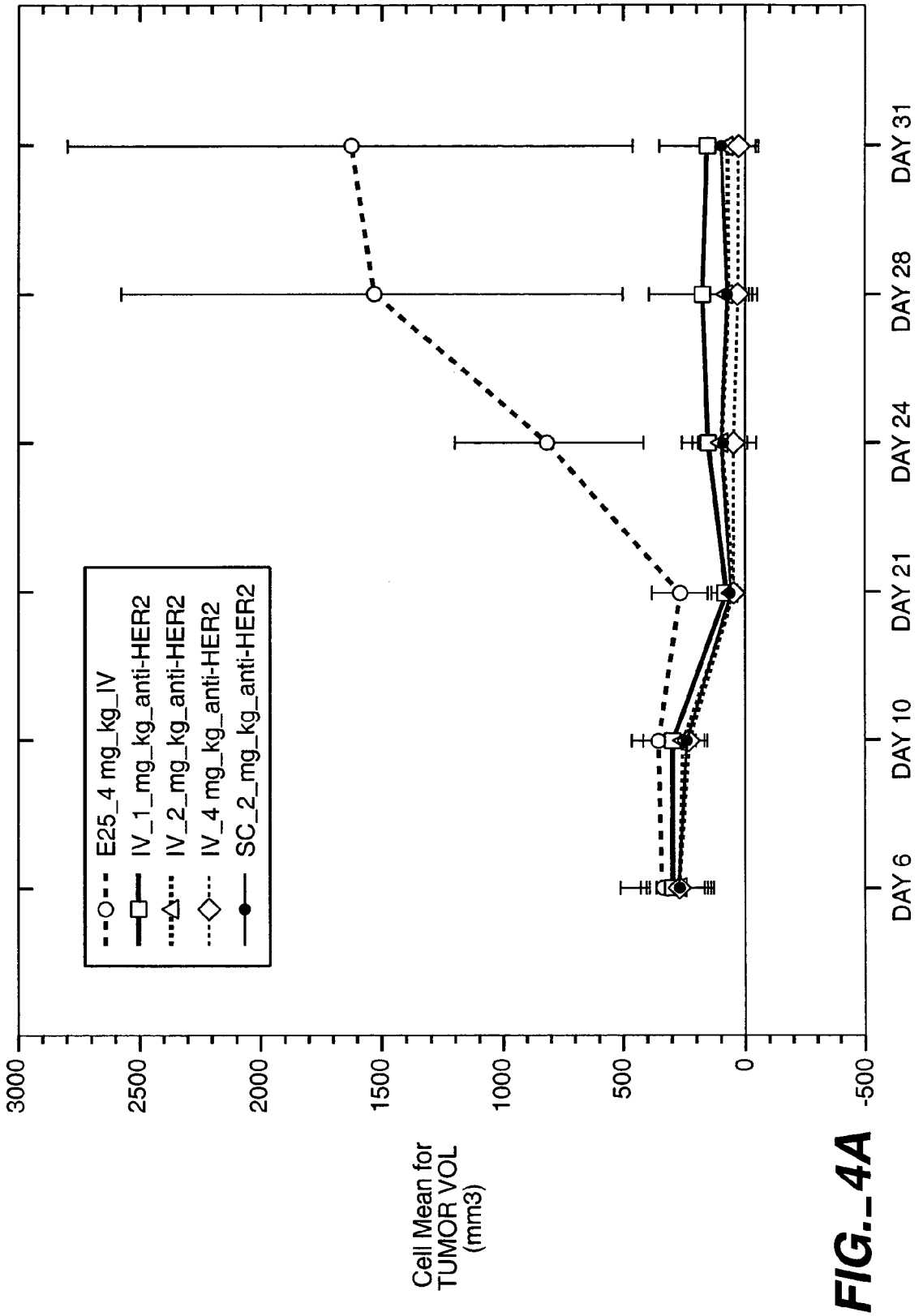
E25_4 mg_kg_IV
IV_1_mg_kg_anti-HER2
IV_2_mg_kg_anti-HER2
IV_4 mg_kg_anti-HER2
SC_2_mg_kg_anti-HER2
Cell Mean for TUMOR VOL (mm3)
3000
2500
2000
1500
1000
500
0
-500
DAY 6
DAY 10
DAY 21
DAY 24
DAY 28
DAY 31

FIG._4A

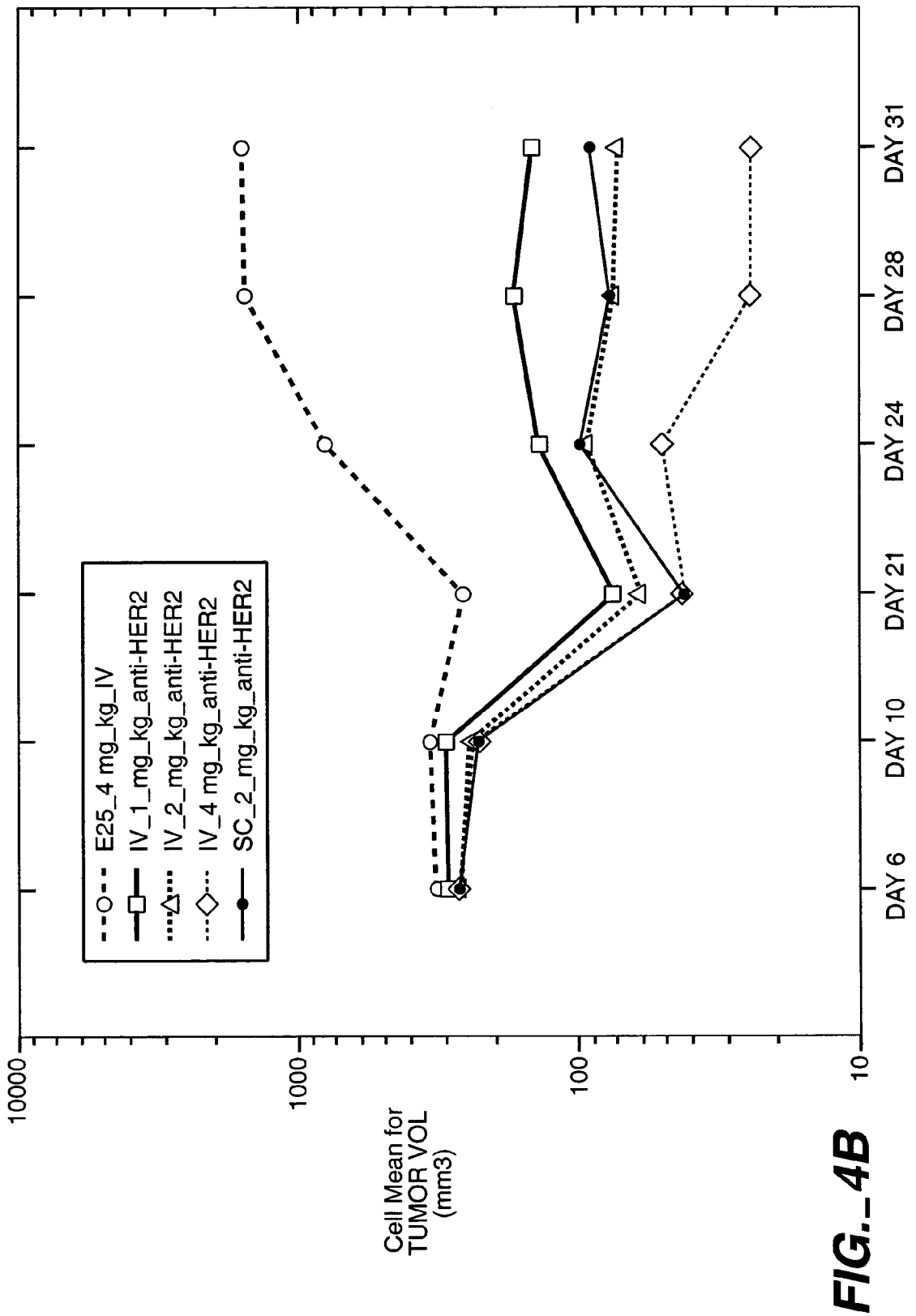
E25_4 mg_kg_IV
IV_1_mg_kg_anti-HER2
IV_2_mg_kg_anti-HER2
IV_4 mg_kg_anti-HER2
SC_2_mg_kg_anti-HER2
Cell Mean for TUMOR VOL (mm3)
10000
1000
100
10
DAY 6
DAY 10
DAY 21
DAY 24
DAY 28
DAY 31

FIG._4B

VARIABLE LIGHT

```
          1       10        20          30              40
2C4       DTVMTQSHKIMSTSVGDRVSITC [KASQDVSIGVA----] WYQQRP
           **    **** *      *                        *
574       DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA----] WYQQKP
                                   *   *  ****
hum kI    DIQMTQSPSSLSASVGDRVTITC [RASQSVSTSSYSYMH] WYQQKP

                    50          60        70        80
2C4       GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA
           **                   *  *         *    * *
574       GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISSLQP
                     *  ****
hum kI    GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP

                   90          100
2C4       EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:10)
            * *                 *   *
574       EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:12)
                      ***
hum kI    EDFATYYC [QQYNSLPYT] FGQGTKVEIK (SEQ ID NO:14)
```

FIG._5A

VARIABLE HEAVY

```
          1       10        20          30          40
2C4       EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS
              **  **  *  * ***  **                   *
574       EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA
                                       ** * *
humIII    EVQLVESGGGSVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

                    50        60          70        80
2C4       HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM
          *  *   **                     *** *    **** *
574       PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL
                  *  ******  ** ****       *. * *
humIII    PGKGLEWVS [VISGDGGSTYYADSVKG] RFTISRDDSKNTLYL

                     90         100           110
2C4       ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLVTSS (SEQ ID NO:11)
          ***  **                              *
574       QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTLVTVSS (SEQ ID NO:13)
                            ** ***
humIII    QMNSLRAEDTAVYYCAR [GRGGGS--DY] WGQGTLVTVSS (SEQ ID NO:15)
```

FIG._5B

DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES

RELATED APPLICATIONS

This application is divisional of U.S. Ser. No. 09/648,067 filed Aug. 25, 2000 (now U.S. Pat. No. 6,627,196), which claims priority under 35 USC 119(e) to provisional application Nos. 60/151,018, filed Aug. 27, 1999 and 60/213,822, filed Jun. 23, 2000, the contents of which are incorporated heroin by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2 or disorders expressing epidermal growth factor receptor (EGFR), comprising administering to a human or animal presenting the disorders a therapeutically effective amount of an antibody that binds ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 or expressing EGFR, where the treatment is with an anti-ErbB2 antibody administered by front loading the dose of antibody during treatment by intravenous and/or subcutaneous administration. The invention optionally includes treatment of cancer in a human patient with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, a taxoid. The taxoid may be, but is not limited to paclitaxel or docetaxel. The invention further includes treatment of cancer in a human patient with a combination of anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, an anthracycline derivative. Optionally, treatment with a combination of anti-ErbB2 and an anthracycline derivative includes treatment with an effective amount of a cardioprotectant. The present invention further concerns infrequent dosing of anti-ErbB2 antibodies.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor ($p185^{HER2}$) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237:78-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) a inhibits colony formation of these cells. In Drebin et al. *PNAS* (*USA*) 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. Al of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994. Hudziak et al., *Mol. Cell. Biol.* 9(3): 1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize $p185^{erbB2}$-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells

was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (*USA*) 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth, whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2, HERCEPTIN®, or HERCEPTIN® anti-ErbB2 antibody) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]). The recommended initial loading dose for HERCEPTIN® is 4 mg/kg administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg and can be administered as a 30-minute infusion if the initial loading dose is well tolerated.

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198:165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Supp11):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the discovery that an early attainment of an efficacious target trough serum concentration by providing an initial dose or doses of anti-ErbB2 antibodies followed by subsequent doses of equal or smaller amounts of antibody (greater front loading) is more efficacious than conventional treatments. The efficacious target trough serum concentration is reached in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The target serum concentration is thereafter maintained by the administration of maintenance doses of equal or smaller amounts for the remainder of the treatment regimen or until suppression of disease symptoms is achieved.

The invention further concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of an anti-ErbB2 antibody subcutaneously. Preferably, the initial dose (or doses) as well as the subsequent maintenance dose or doses are administered subcutaneously. Optionally, where the patient's tolerance to the anti-ErbB2 antibody is unknown, the initial dose is administered by intravenous infusion, followed by subcutaneous administration of the maintenance doses if the patient's tolerance for the antibody is acceptable.

According to the invention, the method of treatment involves administration of an initial dose of anti-ErbB2 antibody of more than approximately 4 mg/kg, preferably more than approximately 5 mg/kg. The maximum initial dose or a subsequent dose does not exceed 50 mg/kg, preferably does not exceed 40 mg/kg, and more preferably does not exceed 30 mg/kg. Administration is by intravenous or subcutaneous administration, preferably intravenous infusion or bolus injection, or more preferably subcutaneous bolus injection. The initial dose may be one or more administrations of drug sufficient to reach the target trough serum concentration in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less.

According to the invention, the initial dose or doses is/are followed by subsequent doses of equal or smaller amounts of antibody at intervals sufficiently close to maintain the

trough serum concentration of antibody at or above an efficacious target level. Preferably, an initial dose or subsequent dose does not exceed 50 mg/kg, and each subsequent dose is at least 0.01 mg/kg. Preferably the amount of drug administered is sufficient to maintain the target trough serum concentration such that the interval between administration cycles is at least one week. Preferably the trough serum concentration does not exceed 2500 μg/ml and does not fall below 0.01 μg/ml during treatment. The front loading drug treatment method of the invention has the advantage of increased efficacy by reaching a target serum drug concentration early in treatment. The subcutaneous delivery of maintenance doses according to the invention has the advantage of being convenient for the patient and health care professionals, reducing time and costs for drug treatment. Preferably, the initial dose (or the last dose within an initial dose series) is separated in time from the first subsequent dose by 4 weeks or less, preferably 3 weeks or less, more preferably 3 weeks or less, most preferably 1 week or less.

In an embodiment of the invention, the initial dose of anti-ErbB2 is 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous administration, such as intravenous infusion or subcutaneous bolus injection. The subsequent maintenance doses are 2 mg/kg delivered once per week by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. The choice of delivery method for the initial and maintenance doses is made according to the ability of the animal or human patient to tolerate introduction of the antibody into the body. Where the antibody is well-tolerated, the time of infusion may be reduced. The choice of delivery method as disclosed for this embodiment applies to all drug delivery regimens contemplated according to the invention.

In another embodiment, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In still another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

In yet another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

In another embodiment, the invention includes initial doses of at least 1 mg/kg, preferably 4 mg/kg, anti-ErbB2 antibody on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In another embodiment, the invention includes an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

In still another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is 2-3 times per week for 3 weeks. In one embodiment of the invention, each dose is approximately 25 mg/kg or less for a human patient, preferably approximately 10 mg/kg or less. This 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

In another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The method of the invention may further comprise administration of a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form. Other preferred ErbB2-binding antibodies include, but are not limited to, antibodies 7C2, 7F3, and 2C4, preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

The present application also provides a method of therapy involving infrequent dosing of an anti-ErbB2 antibody. In particular, the invention provides a method for the treatment of cancer (e.g. cancer characterized by overexpression of the ErbB2 receptor) in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). For instance, the antibody may be administered about every three weeks, about two to about 20 times, e.g. about six times. The first dose and subsequent dose may each be from about 2 mg/kg to about 16 mg/kg; e.g. from about 4 mg/kg to about 12 mg/kg; and optionally from about 6 mg/kg to about 12 mg/kg. Generally, two or more subsequent doses (e.g. from about two to about ten subsequent doses) of the antibody are administered to the patient, and those subsequent doses are preferably separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). The two or more subsequent doses may each be from about 2 mg/kg to about 16 mg/kg; or from about 4 mg/kg to about 12 mg/kg; or from about 6 mg/kg to about 12 mg/kg. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, and a package insert containing instructions to administer the antibody according to such methods.

The presently described dosing protocols may be applied to other anti-ErbB antibodies such as anti-epidermal growth factor receptor (EGFR), anti-ErbB3 and anti-ErbB4 antibodies. Thus, the invention provides a method for the treatment of cancer in a human patient, comprising administering an effective amount of an anti-ErbB antibody to the human patient, the method comprising administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB antibody; and administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose. Alternatively, or additionally, the invention pertains to a method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an

anti-ErbB antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB antibody, and a package insert containing instructions to administer the antibody according to such methods.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition. According to the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody at an initial dose of 5 mg/kg followed by the same or smaller subsequent dose or doses. In another embodiment of the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody subcutaneously for at least one of the doses, preferably for all of the subsequent doses following the initial dose, most preferably for all doses.

In a further aspect, the invention provides a method of treating ErbB2 expressing cancer in a human patient comprising administering to the patient effective amounts of an anti-ErbB2 antibody and a chemotherapeutic agent. In one embodiment of the invention, the chemotherapeutic agent is a taxoid including, but not limited to, paclitaxel and docetaxel. In another embodiment, the chemotherapeutic agent is an anthracyline derivative including, but not limited to, doxorubicin or epirubicin. In still another embodiment of the invention, treatment with an anti-ErbB2 antibody and an anthracycline derivative further includes administration of a cardioprotectant to the patient. In still another embodiment, an anthracycline derivative is not administered to the patient with the anti-ErbB2 antibody. One or more additional chemotherapeutic agents may also be administered to the patient. The cancer is preferably characterized by overexpression of ErbB2.

The invention further provides an article of manufacture comprising a container, a composition within the container comprising an anti-ErbB2 antibody and a package insert instructing the user of the composition to administer the anti-ErbB2 antibody composition and a chemotherapeutic agent to a patient. In another embodiment, the chemotherapeutic agent is other than an anthracycline, and is preferably a taxoid, such as TAXOL®. In still another embodiment, the chemotherapeutic agent is an anthracycline, including but not limited to, doxorubicin or epirubicin. In yet another embodiment, the chemotherapeutic agent is an anthracycline and the package insert further instructs the user to administer a cardioprotectant.

The methods and compositions of the invention comprise an anti-ErbB2 antibody and include a humanized anti-ErbB2 antibody. Thus, the invention further pertains to a composition comprising an antibody that binds ErbB2 and the use of the antibody for treating ErbB2 expressing cancer, e.g., ErbB2 overexpressing cancer, in a human. The invention also pertains to the use of the antibody for treating EGFR expressing cancer. Preferably the antibody is a monoclonal antibody 4D5, e.g., humanized 4D5 (and preferably huMAb4D5-8 (HERCEPTIN® anti-ErbB2 antibody); or monoclonal antibody 2C4, e.g., humanized 2C4. The antibody may be an intact antibody (e.g., an intact IgG_1 antibody) or an antibody fragment (e.g., a Fab, $F(ab')_2$, diabody, and the like). The variable light chain and variable heavy chain regions of humanized anti-ErbB2 antibody 2C4 are shown in FIGS. **5**A and **5**B.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative Mabs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 24 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. **2** depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO:1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

FIG. **3** is a graph of anti-ErbB2 antibody (HERCEPTIN®) trough serum concentration (μg/ml, mean±SE, dark circles) by week from week 2 through week 36 for ErbB2 overexpressing patients treated with HERCEPTIN® anti-ErbB2 antibody at 4 mg/kg initial dose, followed by 2 mg/kg weekly. The number of patients at each time point is represented by "n" (white squares).

FIG. **4**A is a linear plot of tumor volume changes over time in mice treated with HERCEPTIN® anti-ErbB2 antibody. FIG. **4**B is a semi-logarithmic plot of the same data as in FIG. **4**A such that the variation in tumor volume for the treated animals is observed more readily.

FIGS. **5**A and **5**B depict alignments of the amino acid sequences of the variable light (V_L) (FIG. **5**A) and variable heavy (V_H) (FIG. **5**B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 10 and 11, respectively); V_L and V_H domains of humanized Fab version 574 (SEQ ID Nos. 12 and 13, respectively), and human V_L and V_H consensus frameworks (hum κ1, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 14 and 15, respectively). Asterisks identify differences between humanized Fab version 574 and murine monoclonal antibody 2C4 or between humanized Fab version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets. Humanized Fab version 574, with the changes ArgH71Val, AspH73Arg and IleH69Leu, appears to have binding restored to that of the original chimeric 2C4 Fab fragment. Additional FR and/or CDR residues, such as L2, L54, L55, L56, H35 and/or H48, may be modified (e.g. substituted as follows—IleL2Thr; ArgL54Leu; TyrL55Glu;

ThrL56Ser; AspH35Ser; and ValH48Ile) in order to further refine or enhance binding of the humanized antibody. Alternatively, or additionally, the humanized antibody may be affinity matured in order to further improve or refine its affinity and/or other biological activities.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

An "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3 and ErbB4 receptors as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881-914 (1987), including variants thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS* (*USA*) 87:4207-4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.).

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS* (*USA*) 86:9193-9197 (1989), including variants thereof Examples of antibodies which bind HER3 are described in U.S. Pat. No. 5,968,511 (Akita and Sliwkowski), e.g. the 8B8 antibody (ATCC HB 12070) or a humanized variant thereof

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-475 (1993), including variants thereof such as the HER4 isoforms disclosed in WO 99/19488.

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms "ErbB2" "c-Erb-B2" and "HER2" when used herein refer to the human protein, and "erbB2," "c-erb-B2," and "her2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS* (*USA*) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. **1**) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. **1**, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N-terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, E d Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO: 2).

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the "PI uptake assay in BT474 cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the "cell" is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5

to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science,* 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.,* 14(3):1909-1919 (1994); and Marchionni et al., *Nature,* 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177-244}$).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20):14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. 1, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these maybe further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ,

respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature,* 321:522-525 (1986); Reichmann et al., *Nature,* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.,* 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of

treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see Example 1, below). Therapeutically effective amount also refers to a target serum concentration, such as a trough serum concentration, that has been shown to be effective in suppressing disease symptoms when maintained for a period of time.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidaniine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The *Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (W B Saunders:

Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions,* 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g.,an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

The term "serum concentration," "serum drug concentration," or "serum HERCEPTIN® anti-ErbB2 antibody concentration" refers to the concentration of a drug, such as HERCEPTIN® anti-ErbB2 antibody, in the blood serum of an animal or human patient being treated with the drug. Serum concentration of HERCEPTIN® anti-ErbB2 antibody, for example, is preferably determined by immunoassay. Preferably, the immunoassay is an ELISA according to the procedure disclosed herein.

The term "peak serum concentration" refers to the maximal serum drug concentration shortly after delivery of the drug into the animal or human patient, after the drug has been distributed throughout the blood system, but before significant tissue distribution, metabolism or excretion of drug by the body has occurred.

The term "trough serum concentration" refers to the serum drug concentration at a time after delivery of a previous dose and immediately prior to delivery of the next subsequent dose of drug in a series of doses. Generally, the trough serum concentration is a minimum sustained efficacious drug concentration in the series of drug administrations. Also, the trough serum concentration is frequently targeted as a minimum serum concentration for efficacy because it represents the serum concentration at which another dose of drug is to be administered as part of the treatment regimen. If the delivery of drug is by intravenous administration, the trough serum concentration is most preferably attained within 1 day of a front loading initial drug delivery. If the delivery of drug is by subcutaneous administration, the peak serum concentration is preferably attained in 3 days or less. According to the invention, the trough serum concentration is preferably attained in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, most preferably in 1 week or less, including 1 day or less using any of the drug delivery methods disclosed herein.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human patient over a period of time greater than approximately 5 minutes, preferably between approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human patient, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery

from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human patient, preferably within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human patient, wherein the pump delivers a predetermined amount of drug for a predetermined period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human patient, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example, by pinching or drawing the skin up and away from underlying tissue.

The term "front loading" when referring to drug administration is meant to describe an initially higher dose followed by the same or lower doses at intervals. The initial higher dose or doses are meant to more rapidly increase the animal or human patient's serum drug concentration to an efficacious target serum concentration. According to the present invention, front loading is achieved by an initial dose or doses delivered over three weeks or less that causes the animal's or patient's serum concentration to reach a target serum trough concentration. Preferably, the initial front loading dose or series of doses is administered in two weeks or less, more preferably in 1 week or less, including 1 day or less. Most preferably, where the initial dose is a single dose and is not followed by a subsequent maintenance dose for at least 1 week, the initial dose is administered in 1 day or less. Where the initial dose is a series of doses, each dose is separated by at least 3 hours, but not more than 3 weeks or less, preferably 2 weeks or less, more preferably 1 week or less, most preferably 1 day or less. To avoid adverse immune reaction to an antibody drug such as an anti-ErbB2 antibody (e.g., HERCEPTIN® anti-ErbB2 antibody) in an animal or patient who has not previously been treated with the antibody, it may be preferable to deliver initial doses of the antibody by intravenous infusion. The present invention includes front loading drug delivery of initial and maintenance doses by infusion or bolus administration, intravenously or subcutaneously.

Published information related to anti-ErbB2 antibodies includes the following issued patents and published applications: PCT/US89/0005 1, published Jan. 5, 1989; PCT/US90/02697, published May 18, 1990; EU 0474727 issued Jul. 23, 1997; DE 69031120.6, issued Jul. 23, 1997; PCT/US97/18385, published Oct. 9, 1997; SA 97/9185, issued Oct. 14, 1997; U.S. Pat. No. 5,677,171, issued Oct. 14, 1997; U.S. Pat. No. 5,720,937, issued Feb. 24, 1998; U.S. Pat. No. 5,720,954, issued Feb. 24, 1998; U.S. Pat. No. 5,725,856, issued Mar. 10, 1998; U.S. Pat. No. 5,770,195, issued Jun. 23, 1998; U.S. Pat. No. 5,772,997, issued Jun. 30, 1998; PCT/US98/2626, published Dec. 10, 1998; and PCT/US99/06673, published Mar. 26, 1999, each of which patents and publications is herein incorporated by reference in its entirety.

II. Production of anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al., *PNAS* (*USA*) 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl^2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypox-

anthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352: 624-628 (1991) and Marks et al., *J. Mol. Biol,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al. *Science,* 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable

three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al. *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10: 163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO 89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as his (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang et al., *Proc. Natl Acad. Sci. USA,* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al., *J. National Cancer Inst.* 81(19)1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO 81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature*

328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature,* 312: 604-608 [1984]).

(xi) Antibody-Salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g. by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Determination of anti-ErbB2 Antibody Concentration in Serum

The following non-limiting assay is useful for determining the presence of and to quantitate the amount of specific rhuMAb HER2 (humanized anti-$p185^{HER2}$ monoclonal antibody, including HERCEPTIN® anti-ErbB2 antibody) in a body fluid of a mammal including, but not limited to, serum, amniotic fluid, milk, umbilical cord serum, ocular aqueous and vitreous liquids, and ocular vitreous gel.

Plate Binding Activity Assay for rhuMAb HER2 (Humanized Anti-p185^{HER2} Monoclonal Antibody

The method of assaying rhuMAb HER2 described herein is meant as an example of such a method and is not meant to be limiting. A standardized preparation of rhuMAb HER2 (Genentech, Inc., South San Francisco, Calif.), controls, and serum samples were diluted with Assay Diluent (PBS/0.5% BSA/0.05% Polysorbate 20/0.01% Thimerosal). The dilutions of standardized rhuMAb HER2 were prepared to span a range of concentrations useful for a standard curve. The samples were diluted to fall within the standard curve.

An aliquot of Coat Antigen in Coating buffer (recombinant p185^{HER2} (Genentech, Inc.) in 0.05 M sodium carbonate buffer) was added to each well of a microtiter plate and incubated at 2-8° C. for 12-72 hours. The coating solution was removed and each well was washed six times with water, then blotted to remove excess water.

An aliquot of Assay Diluent was added to each well and incubated for 1-2 hours at ambient temperature with agitation. The wells were washed as in the previous step.

Aliquots of diluted standard, control and sample solutions were added to the wells and incubated at ambient temperature for 1 hour with agitation to allow binding of the antibody to the coating antigen. The wells are washed again with water as in previous steps.

Horse radish peroxidase-conjugate (HRP-conjugate, Goat anti-human IgG Fc conjugated to horseradish peroxidase; Organon Teknika catalog #55253 or equivalent) was diluted with Assay Diluent to yield an appropriate optical density range between the highest and lowest standards. An aliquot of the HRP-conjugate solution was added to each well and incubated at ambient temperature for I hour with agitation. The wells were washed with water as in previous steps.

An aliquot of Substrate Solution (o-phenylenediamine (OPD) 5 mg tablet (Sigma P6912 or equivalent) in 12.5 ml 4 mM H_2O_2 in PBS) was added to each well and incubated for a sufficient period of time (approximately 8-10 minutes) in the dark at ambient temperature to allow color development. The reaction was stopped with an aliquot of 4.5 N sulfuric acid. Optical density was read at 490-492 nm for detection absorbance and 405 nm for reference absorbance. The standard curve data are plotted and the results for the controls and samples are determined from the standard curve.

IV. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16 th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial growth factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16 th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

V. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disor-

ders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

The treatment of the present invention involves the administration of an anti-ErbB2 antibody to an animal or human patient, followed at intervals by subsequent doses of equal or smaller doses such that a target serum concentration is achieved and maintained during treatment. Preferably, maintenance doses are delivered by bolus delivery, preferably by subcutaneous bolus administration, making treatment convenient and cost-effective for the patient and health care professionals.

Where combined administration of a chemotherapeutic agent (other than an antracycline) is desired, the combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It maybe desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial growth factor (VEGF). Alternatively, or additionally, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. The ErbB2 antibody may be co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration, or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

In addition to the above therapeutic regimens, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of anti-ErbB2 antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Where the treatment involves a series of treatments, the initial dose or initial doses are followed at daily or weekly intervals by maintenance doses. Each maintenance dose provides the same or a smaller amount of antibody compared to the amount of antibody administered in the initial dose or doses.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy is easily monitored by conventional techniques and assays.

According to the invention, dosage regimens may include an initial dose of anti-ErbB2 of 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous infusion, followed by subsequent weekly maintenance doses of 2 mg/kg by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. Where the antibody is well-tolerated by the patient, the time of infusion may be reduced.

Alternatively, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

Another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

Still another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

As an alternative regimen, initial doses of 4 mg/kg anti-ErbB2 antibody may be administered on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

An additional regimen involves an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

Alternatively, the invention may include a cycle of dosing in which delivery of anti-ErbB2 antibody is 2-3 times per week for 3 weeks. The 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The invention further includes a cyclic dosage regimen in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms. Further information about suitable dosages is provided in the Examples below.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container

holds a composition which is effective for treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture may comprise a package inserts with instructions for use, including, e.g., a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin or epirubicin.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRT 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLES

Example 1

Preparation and Efficacy of HERCEPTIN® Anti-ErbB2 Antibody Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 $IgG_1\kappa$ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 5o:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2-3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al., *Proc. Natl. Acad. Sci.* (*USA*) 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN® anti-ErbB2 antibody). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG_1 (IgG_1) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for $p185^{HER2}$ (Dillohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of $p185^{HER2}$, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® anti-ErbB2 antibody is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

- Metastatic breast cancer
- Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to overexpress ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for $p185^{HER2}$].
- Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

- The ability to understand and willingness to sign a written informed consent form
- Women>18 years
- Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

- Prior cytotoxic chemotherapy for metastatic breast cancer
- Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.
- Concomitant malignancy that has not been curatively treated
- A performance status of <60% on the Kamofsky scale

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® anti-ErbB2 antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regimens for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® anti-ErbB2 antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m^2) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paciltaxel (TAXOL®) was given at a dose of 175 mg/m^2 over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H_2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed. 2), New York, N.Y., Wiley, 1981, pp 13-17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN® anti-ErbB2 antibody, without increase in overall severe adverse events (AE):

TABLE 1

HERCEPTIN ® Anti-ErbB2 Antibody Efficacy

	Enrolled	TTP(months)	RR(%)	AE(%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p < 0.001 by log-rank test;
**p < 0.01 by X^2 test;
CRx: chemotherapy;
AC: anthracycline/cyclophosphamide treatment;
H: HERCEPTIN ® anti-ErbB2 antibody;
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade ¾) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor treatment with HERCEPTIN® anti-ErbB2 antibody and paclitaxel (TAXOL®) where a combined treatment regimen is desired.

Example 2

Pharmacokinetic and Pharmacodynamic Properties of Anti-ErbB2 Antibody (HERCEPTIN®)

HERCEPTIN® anti-ErbB2 antibody was administered by intravenous infusion to human patients selected according to the criteria provided in Example 1. An initial dose of 4 mg/kg HERCEPTIN® anti-ErbB2 antibody was delivered by intravenous infusion, followed by subsequent i.v. infu-

sions of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody weekly for several weeks. Two hundred thirteen patients began this treatment regimen and serum drug concentration was obtained beyond 8 weeks for fewer than 90 patients as selective discontinuation of patients with rapidly progressing disease occurred. Of the 213 patients who began treatment, serum trough concentration data were available for 80 patients at Week 12, for 77 patients at Week 16, for 44 patients at Week 20, for 51 patients at Week 24, for 25 patients at Week 28, for 23 patients at Week 32, and for 37 patients at Week 36.

HERCEPTIN® Anti-ErbB2 Antibody Trough Serum Concentrations for Weeks 0-36

The HERCEPTIN® anti-ErbB2 antibody trough serum concentrations (μg/ml, mean±SE) from Week 2 through Week 36 are plotted in FIG. **3** (dark circles). The number of patients was fairly constant because data from patients discontinued from the program due to rapidly progressing disease were excluded from this analysis. Trough serum concentrations tended to increase through Week 12 and tended to plateau after that time.

HERCEPTIN® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for Weeks 1-8

Some HERCEPTIN® anti-ErbB2 antibody serum concentration data were available for 212 of the original 213 patients. Trough and peak serum concentration data reflecting the first HERCEPTIN® anti-ErbB2 antibody infusion were available for 195 of the 212 patients. For the seventh infusion, trough serum concentration data were available for 137/212 patients and peak serum concentration data were available for 114/212 patients. Table 2 presents a summary of statistics from trough and peak serum concentrations for the first 8 weeks of treatment. Peak samples were drawn shortly after the end of HERCEPTIN® anti-ErbB2 antibody administration; trough samples were drawn prior to the subsequent dose (i.e., 1 week later). Serum concentrations of HERCEPTIN® anti-ErbB2 antibody were determined as disclosed herein.

TABLE 2

HERCEPTIN ® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for the First 8 Weeks of Treatment (μg/ml)

	Dose Number	n	Mean	SD	Minimun	Maximum
Peak	1	195	100.3	35.2	30.7	274.6
Trough		195	25.0	12.7	0.16	60.7
Peak	2	190	74.3	31.3	20.8	307.9
Trough		167	30.4	16.0	0.2	74.4
Peak	3	167	75.3	26.8	16.1	194.8
Trough		179	33.7	17.9	0.2	98.2
Peak	4	175	80.2	26.9	22.2	167
Trough		132	38.6	20.1	0.2	89.4
Peak	5	128	85.9	29.2	27.8	185.8
Trough		141	42.1	24.8	0.2	148.7
Peak	6	137	87.2	32.2	28.9	218.1
Trough		115	43.2	24.0	0.2	109.9
Peak	7	114	89.7	32.5	16.3	187.8
Trough		137	48.8	24.9	0.2	105.2
Peak	8	133	95.6	35.9	11.4	295.6

The data in Table 2 suggest that there was an increase in trough serum concentration over time. Of the many patients studied, there were 18 patients for whom the trough concentrations did not exceed 20 μg/ml from Week 2 through Week 8. A HERCEPTIN® anti-ErbB2 antibody trough serum concentration of 20 μg/ml was nominally targeted for these studies based on prior pharmacologic studies in animals and exploratory analyses in clinical trials.

Patient response status was evaluated relative to serum concentration of HERCEPTIN® anti-ErbB2 antibody. For this purpose, mean serum concentration (an average of troughs and peaks) was calculated for various times and patient response status (where the patient response status was determined by an independent Response Evaluation Committee). The increase in serum concentration between Weeks 2 and 8 appeared to be greater in responders than in nonresponders, suggesting that there is a relationship between response status and HERCEPTIN® anti-ErbB2 antibody serum concentration. A statistical analysis (analysis of variance) of trough serum concentration values at Week 2 and an average of Weeks 7 and 8 in relation to response status indicated a highly significant relationship between response status and average trough of Weeks 7 and 8 ($p<0.001$). The results indicated that there was a significant difference between the trough serum concentration (average troughs of Weeks 7 and 8) in the responders and nonresponders: trough concentrations were 60±20 μg/ml in the responders versus 44±25 μg/ml in the nonresponders (mean±SD). HER2 overexpression level and type of metastatic sites were associated with significant differences in trough serum concentrations. At Week 2, patients with 2+ HER2 overexpression had significantly higher trough serum concentrations (n=40, mean=28.8 μg/ml, SD=10.4) compared with patients with 3+ HER2 overexpression (n=155, mean=24.1 μg/ml, SD=13.1). This difference in the average trough serum concentrations for Weeks 7 and 8 was no longer statistically significant. Further, at Week 2, patients with superficial disease had significantly higher trough serum concentrations (n=12, mean 34.1 μg/ml, SD=12.0) compared with patients with visceral disease (n=183, mean=24.4 μg/ml, SD=12.6). This difference in the average trough serum concentrations for Weeks 7 and 8 was significant. These data indicate that the rise in trough serum concentrations between Weeks 2 and 7/8 occurs for human patients with various disease profiles.

In a subsequent, similarly designed study, human breast cancer patients were treated with a loading dose of 8 mg/kg followed by maintenance doses of4 mg/kg weekly. The results of this preliminary human study indicated that an 8 mg/kg load:4 mg/kg weekly maintenance regimen was efficacious in reducing tumor volume in the patients.

The data disclosed in this Example indicate that front loading of antibody, such that a target serum concentration is reached more quickly, may be associated with improved outcomes.

Example 3

I.V. Bolus Delivery and Subcutaneous Infusion of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

The efficacy of infusion or bolus delivery of humanized anti-ErbB2 antibody (HERCEPTIN®, see Example 1 for preparation), either by intravenous injection or subcutaneous injection, was examined. The purpose of the study was to ask whether subcutaneous delivery was feasible and whether the convenient subcutaneous bolus delivery was useful in treating metastatic breast cancer in animals inoculated with a cell line that overexpresses the HER2 gene. The results, detailed below, show that i.v. and s.c. infusion and bolus delivery are feasible treatment methodologies.

A study in a nude mouse xenograft model, which incorporates a human breast cancer cell line that naturally overexpresses the HER2 gene (BT-474M1, derived from BT-474 cells, ATCC Accession number HTB-20), comparing tumor volume as a function of i.v. bolus versus s.c. infusion was performed as follows. In the first study athymic nude nu nu

7-9 week old female mice were obtained from Taconic Inc (Germantown, N.Y.). To initiate tumor development, each mouse was inoculated subcutaneously with 3×10^6 BT474M1 cells suspended in Matrigel™. When tumor nodules reached a volume of approximately 100 mm^3, animals were randomized to 4 treatment groups. The groups were treated according to Table 3.

TABLE 3

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Infusion

Group, Dose, Antibody	Target Serum Conc. µg/ml	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose
1 - Control, rhuMAb E25	20	IV LD and SC infusion	2.20	0.250 mg/ml (infusate)
2 - Low Dose SC rhuMAb HER2	1	IV LD and SC infusion	0.313	0.050 mg/ml (infusate)
3 - High Dose SC rhuMAb HER2	20	IV LD and SC infusion	6.25	1.00 mg/ml (infusate)
4 - IV Multi-Dose rhuMAb HER2	20 (trough)	IV LD and MD	4.00	2 mg/kg/week (IV bolus)

Serum Conc. = concentration in serum. LD = loading dose. MD = maintenance dose.
Infusate concentration was calculated to achieve targeted serum concentration using Alzet ® osmotic minipumps (Alza Corp., Palo Alto, CA).

Animals were exposed to estrogen by subcutaneous sustained release estrogen pellet 9 days before the start of dosing to promote growth of grafted tumor cells. The animals were inoculated with the BT474M 1 cells 8 days before the beginning of treatment and tumors were allowed to grow. The animals were then treated with nonrelevant antibody E25 (non-specific for HER2 receptor, but a member of the monoclonal IgG class) or test antibody HERCEPTIN® anti-ErbB2 anitbody as indicated in Table 3. The dosage levels were selected to achieve target serum concentrations of HERCEPTIN®, either 1 µg/ml or 20 µg/ml, by subcutaneous pump infusion or by i.v. bolus delivery. The study groups were treated until day 35. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly (just prior to dosing for Group 4) using 3 mice/group/time point. The anti-ErbB2 antibody concentration was determined according to the method disclosed herein involving standard techniques. Tumor volumes were measured two days before dosing began and twice per week from day 6 to day 35 in the study for which data is tabulated below. Tumors were measured in three dimensions and volumes were expressed in mm^3. Efficacy was determined by a statistical comparison (ANOVA) of tumor volumes of test animals relative to untreated control animals.

As shown in Table 4, below, treatment of the BT474M 1 tumor-bearing mice with HERCEPTIN® anti-ErbB2 antibody by the indicated dosage methods significantly inhibited the growth of the tumors. All HERCEPTIN®-treated groups showed similar inhibition of tumor growth relative to the control group. No dose-response was observed.

TABLE 4

Comparison of S.C. Infusion and I.V. Bolus Delivery

Treatment Group	Tumor Volume (mm^3), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6-Day 35 (n = 13)	HERCEPTIN ® Serum Conc. (µg/ml), Day 27, (n = 3)
control s.c. infusion	764 ± 700	5650 ± 4700	4.16 ± 1.94

TABLE 4-continued

Comparison of S.C. Infusion and I.V. Bolus Delivery

Treatment Group	Tumor Volume (mm^3), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6-Day 35 (n = 13)	HERCEPTIN ® Serum Conc. (µg/ml), Day 27, (n = 3)
s.c. infusion (low dose)	80.6 ± 158	1610 ± 1250	2.11 ± 1.74
s.c. infusion (high dose)	31 ± 75.6	1440 ± 1140	22.1 ± 5.43
i.v. bolus dose*	49.7 ± 95.7	2150 ± 1480	21.7 ± 17.1**

s.c. = subcutaneous delivery; i.v. = intravenous delivery.
*4.0 mg/kg Loading Dose and 2.0 mg/kg/week Maintenance Dose.
**at predose (trough serum concentration immediately prior to a maintenance dose)

The results tabulated above indicate that maintenance of a serum concentration of approximately 2 µg/ml was as effective as a concentration of 20 µg/ml in this study. The results indicated that dosing by subcutaneous infusion was as effective as intravenous bolus dosing and achieved similar trough serum concentrations. The results also indicate that the dose levels studied are at the top of the dose-response curve in this model and that subcutaneous dosing is effective in treating breast cancer tumors. Thus, subcutaneous administration of maintenance doses is feasible as part of a HERCEPTIN® anti-ErbB2 antibody treatment regimen.

Example 4

I.V. Bolus and Subcutaneous Bolus Deliveries of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

Subcutaneous bolus delivery is convenient and cost-effective for the patient and health care professionals. The results of the study disclosed in this example indicate that subcutaneous bolus delivery was as effective as intravenous bolus delivery in reducing breast cell tumor size in a mouse.

This study was set up as disclosed herein in Example 3 for the comparison of intravenous bolus and subcutaneous infusion delivery. A sustained release estrogen implant was inserted subcutaneously one day before tumor cell innoculation as described in Example 3. Six days after tumor cell innoculation, the initial tumor measurement was performed. Seven days after tumor cell innoculation, the first dose of control antibody or HERCEPTIN® anti-ErbB2 antibody was delivered. The animal groups, type of delivery, loading dose and maintenance doses are provided in Table 4. Animals were dosed once weekly for 4 weeks.

TABLE 5

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Bolus Delivery

Group	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose (mg/kg/week)	n
1 - Control rhuMAb E25	IV	8	4	10
2 - rhuMAb HER2	IV	2	1	10
3 - rhuMAb HER2	IV	4	2	10
4 - rhuMAb HER2	IV	8	4	10
5 - rhuMAb HER2	SC	4	2	10

IV = intraveneous; SC = subcutaneous; n = number of animals per group.

The mice were treated according to the information in Table 4 and using the techniques disclosed in Example 3. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly before each weekly i.v. maintenance dose according to the procedure described herein and using standard techniques. The control E25 antibody serum concentration was determined according to standard immunoassay techniques. Table 6 shows the increase in HERCEPTIN® anti-ErbB2 antibody serum concentrations with time.

TABLE 6

IV versus SC Bolus Delivery: Serum HERCEPTIN ® Anti-ErbB2 Antibody Concentration

	Serum Concentration, μg/ml			
Treatment Group (delivery, MD)	Day 0 Mean (SD)	Day 7 Mean (SD)	Day 14 Mean (SD)	Day 21 Mean (SD)
1 - Control rhu MAb E25	0	25.9	34.6	38.5
(IV, 4 mg/kg)	(0)	(8.29)	(11.2)	(14.4)
2 - rhu MAb HER2	0	4.96	8.55	8.05
(IV, 1 mg/kg)	(0)	(3.79)	(5.83)	(4.67)
3 - rhu MAb HER2	0	13.4	18.9	22.6
(IV, 2 mg/kg)	(0)	(9.24)	(12.0)	(9.21)
4 - rhu MAb HER2	0	29.6	37.7	46.2
(IV, 4 mg/kg)	(0)	(13.5)	(14.4)	(13.8)
5 - rhu MAb HER2	0	12.5	16.9	17.6
(SC, 2 mg/kg)	(0)	(7.33)	(10.2)	(10.7)

n = 10 for time points Days 0, 7 and 14. N = 9 for Day 21.

Table 7 shows the relative efficacy of intravenous bolus delivery and subcutaneous bolus delivery for Groups 1-5 having achieved the serum antibody concentrations presented in Table 6. For this study, efficacy was measured as a decrease in tumor volume. Tumor volume was measured twice weekly.

TABLE 7

Efficacy of HERCEPTIN ® Anti-ErbB2 Antibody Measured as a Change in Tumor Volume Comparing Intravenous Bolus and Subcutaneous Bolus Delivery, Mean (SD)

Treatment Group (Delivery, MD)	Tumor Vol. Day 6, mm^3	Tumor Vol. Day 28, mm^3	Tumor Vol. Day 31, mm^3	Day 6-Day 31* Area Under Curve Tumor Vol., mm^3	Tumor Growth Rate on Log (TM + 1)
1-IV Control	321	1530	1630	13600	0.0660
	(190)	(1040)	(1170)	(7230)	(0.0200)
2-IV Herceptin	297	175	151	4690	−0.0505
1 mg/kg	(130)	(215)	(188)	(1400)	(0.142)
3-IV Herceptin	269	75.7	73.6	3510	−0.0608
2 mg/kg	(129)	(92.4)	(84.5)	(1220)	(0.110)
4-IV Herceptin	272	25.3	25.8	2880	−0.0810
4 mg/kg	(117)	(75.9)	(72.9)	(1230)	(0.0859)
5-SC Herceptin	268	76.2	90.4	3230	−0.0304
2 mg/kg	(117)	(98.8)	(105)	(1440)	(0.104)

N = 10 for each data point. TM = tumor measurement. IV = intravenous. SC = subcutaneous. MD = maintenance dose. Tumor Vol. = tumor volume, mm^3.
*Day 17 excluded due to measurement error.
Tumor growth rate calculated on Day 21-Day 31 Log (TM + 1). Area under the curve is the area beneath a plot of tumor volume versus time.

FIGS. **4**A and **4**B are graphical plots of changes in tumor volume over time, some of which data is found in Table 7. FIG. **4**A is a linear plot of tumor volume versus time. FIG. **4**B is a semilogarithmic plot of the same data, allowing the test points be viewed more clearly. The data in Table 7 and FIGS. **4**A and **4**B indicate that, although a dose-related response was not observed between HERCEPTIN-treated groups, dosing by subcutaneous bolus was as effective as intravenous bolus dosing and achieved similar trough serum concentrations.

Example 5

Regimens for Intravenous and Subcutaneous Delivery of Anti-ErbB2 Antibody

According to the invention, methods of anti-ErbB2 antibody (e.g., HERCEPTIN®) delivery comprise greater front loading of the drug to achieve a target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less. According to the invention, this initial dosing is followed by dosing that maintains the target serum concentration by subsequent doses of equal or smaller amount. An advantage of the methods of the invention is that the maintenance dosing may be less frequent and/or delivered by subcutaneous injection, making the treatment regimens of the invention convenient and cost-effective for the patient and medical professionals administering the antibody. In addition, a subcutaneous maintenance dose regimen may be interrupted by intravenous dosing (such as infusion) when the patient's chemotherapy requires delivery of other drugs by intravenous injection.

To test the following dosage regimens, human subjects are selected according to the criteria disclosed in Example 1, above. The number of initial doses is one or more doses sufficient to achieve an efficacious target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The number of maintenance doses may be one or more doses sufficient to achieve suppression of disease symptoms, such as a decrease in tumor volume. The maintenance doses are equal to or smaller than the initial dose or doses, consistent with an object of the invention of administering HERCEPTIN® anti-ErbB2 antibody by regimens providing greater front loading. The specific drug delivery regimens disclosed herein are representative of the invention and are not meant to be limiting.

In one trial, an initial dose of 6 mg/kg, 8 mg/kg, or 12 mg/kg of HERCEPTIN® anti-ErbB2 antibody is delivered to human patients by intravenous or subcutaneous injection. Initial doses (loading doses) are delivered by intravenous infusion or bolus injection or preferably subcutaneous bolus injection. Preferably a target trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10-20 μg/ml is achieved (averaged for all patients in the treatment group) and maintained by subsequent doses of anti-ErbB2 antibody that are equal to or smaller than the initial dose. In one method, a target trough serum concentration is achieved and maintained by once-per-week deliveries of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody by intravenous or subcutaneous injection for at least eight weeks. Alternatively, for this or any dosage regimen disclosed herein, subcutaneous continuous infusion by subcutaneous pump is used to delivery subsequent maintenance doses.

In another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10-20 μg/ml, averaged for an entire treatment group.

In another method, an initial (front loading) dose of 12 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10-20 μg/ml.

In yet another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous infusion or bolus injection, or preferably by subcutaneous bolus injection or infusion. This is followed by administration of 8 mg/kg per week or 8 mg/kg per 2-3 weeks to maintain a trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10-20 μg/ml. Maintenance doses are delivered by intravenous infusion or bolus injection, or preferably by subcutaneous infusion or bolus injection.

In another method, the front loading initial dose is a series of intravenous or subcutaneous injections, for example, one on each of days 1, 2, and 3 of at least 1 mg/kg for each injection (where the amount of anti-ErbB2 antibody delivered by the sum of initial injections is more than 4 mg/kg), followed by maintenance doses of 6 mg/kg once each 3 week interval to maintain a target trough serum concentration (for example, approximately 10-20 μg/ml) of HERCEPTIN® anti-ErbB2 antibody. The maintenance doses are delivered by intravenous infusion or bolus injection or by subcutaneous infusion or subcutaneous bolus injection.

In yet another method, the front loading is by intravenous infusion of at least 1 mg/kg, preferably 4 mg/kg on each of five consecutive days, followed by repeats of this cycle a sufficient number of times to achieve suppression of disease symptoms. Following the initial dose or doses, subsequent doses may be delivered by subcutaneous infusion or bolus injection if tolerated by the patient. Such subcutaneous delivery is convenient and cost-effective for the patient and administering health care professionals.

In still another method, HERCEPTIN® anti-ErbB2 antibody is delivered initially as at least 2 intravenous infusions per week for three weeks, followed by repeats of this cycle to maintain an efficacious trough serum concentration of HERCEPTIN® anti-ErbB2 antibody. The dose is at least 4 mg/kg of anti-ErbB2 antibody, preferably at least 5 mg/kg. The maintenance drug deliveries may be intravenous or subcutaneous.

Where the animal or patient tolerates the antibody during and after an initial dose, delivery of subsequent doses may be subcutaneous, thereby providing greater convenience and cost-effectiveness for the patient and health care professionals.

In animal studies, an initial dose of more than 4 mg/kg, preferably more than 5 mg/kg delivered by intravenous or subcutaneous injection, is followed by subcutaneous bolus injections of 2 mg/kg twice per week (separated by 3 days) to maintain a trough serum concentration of approximately 10-20 μg/ml. In addition, where the animal or patient is known to tolerate the antibody, an initial dose of HERCEPTIN® anti-ErbB2 antibody is optionally and preferably deliverable by subcutaneous bolus injection followed by subcutaneous maintenance injections.

While target serum concentrations are disclosed herein for the purpose of comparing animal studies and human trials, target serum concentrations in clinical uses may differ. The disclosure provided herein guides the user in selecting a front loading drug delivery regimen that provides an efficacious target trough serum concentration.

The methods of the invention disclosed herein optionally include the delivery of HERCEPTIN® anti-ErbB2 antibody in combination with a chemotherapeutic agent (other than an anthrocycline derivative) to achieve suppression of disease symptoms. The chemotherapeutic agent may be delivered with HERCEPTIN® anti-ErbB2 antibody or separately and according to a different dosing schedule. For example, subcutaneous delivery of HERCEPTIN® anti-ErbB2 antibody with TAXOL® is included in the invention. In addition, intravenous or subcutaneous injection of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody, followed by intravenous or subcutaneous injection of 6 mg/kg HERCEPTIN® anti-ErbB2 antibody every 3 weeks is administered in combination with a chemotherapeutic agent, such as a taxoid (e.g. paclitaxel 175 mg/m2 every 3 weeks) or an anthracycline derivative (e.g. doxorubicin 60 mg/m2 or epirubicin 75 mg/m2 every 3 weeks). Optionally, where an anthracycline derivative is administered, a cardioprotectant (e.g. 600 mg/m2 cyclophosphamide every 3 weeks) is also administered. In another combination therapy, anti-ErbB2 antibody is administered in a loading dose of more than 4 mg/kg, preferably more than 5 mg/kg, and more preferably at least 8 mg/kg. The loading dose is followed by maintenance doses of at least 2 mg/kg weekly, preferably 6 mg/kg every 3 weeks. The combination therapy includes administration of a taxoid during treatment with anti-ErbB2 antibody. According to one embodiment of the invention, the taxoid is paclitaxel and is administered at a dose of 70-100 mg/m^2/week. According to another embodiment of the invention, the taxoid is docetaxel and is administered at a dose of 30-70 mg/m^2/week.

Example 6

HERCEPTIN® Administered Intravenously Every Three Weeks in Combination with Paclitaxel

Currently, the recommended dose of HERCEPTIN® is 2 mg/kg once weekly. Patients will be administered HERCEPTIN® every three weeks instead of weekly, along with

paclitaxel (175 mg/m^2 every three weeks). Simulation of the proposed treatment regimen suggests that the trough serum concentrations will be 17 mcg/ml, in the range (10-20 mcg/ml) of the targeted trough serum concentrations from previous HERCEPTIN® IV clinical trials. After the first 12 patients the PK parameters will be assessed, if exposure is felt inadequate, then the dose will be increased to 8 mg/kg every three weeks for the remaining 12 patients.

Inclusion Criteria

1) Females24 18 years of age
2) Histologically confirmed ErbB2 over-expressing metastatic breast cancer
3) Patients who have been newly diagnosed with metastatic disease
4) Have a Karnofsky performance status of 24 70%
5) Give written informed consent prior to any study specific screening procedures with the understanding that the patient has the right to withdraw from the study at any time, without prejudice.

Exclusion Criteria

1) Pregnant or lactating women
2) Women of childbearing potential unless (1) surgically sterile or (2) using adequate measures of contraception such as oral contraceptive, intra-uterine device or barrier method of contraception in conjunction with spermicidal jelly.
3) Clinical or radiologic evidence of CNS metastases.
4) History of any significant cardiac disease
5) LVEF≦50%
6) No prior taxane therapy in any treatment setting.
7) Any of the following abnormal baseline hematologic values:
   Hb less than 9 g/dl
   WBC less than 3.0×10^9/l
   Granulocytes less than 1.5×10^9/l
   Platelets less than 100×10^9/l
8) Any of the following abnormal baseline liver function tests:
   Serum bilirubin greater than 1.5×ULN (upper normal limit)
   ALT and/or AST greater than 2.5×ULN (greater than 4.0×ULN if liver or bone metastasis)
   Alkaline phosphatase greater than 2.5×ULN (greater than 4.0×ULN if liver or bone metastasis)
9) The following abnormal baseline renal function tests:
   serum creatinine greater than 1.5×ULN
10) History of other serious medical conditions that would preclude patient participation in an investigational study.

HERCEPTIN® Loading dose and schedule: 8 mg/kg for first dose. Maintenance dose and schedule: 6 mg/kg every 3 weeks.

Paclitaxel—175 mg/m^2 IV every 3 weeks×6 cycles as a 3-hour infusion.

NOTE: On the first cycle of treatment, paclitaxel will be dosed 8 hours prior to HERCEPTIN® to determine the PK of paclitaxel alone. HERCEPTIN® will be administered 8 hours post-paclitaxel for the 1st cycle only. In subsequent treatment cycles, HERCEPTIN® will be administered prior to paclitaxel.

The total duration of this study is 18 weeks. Study subjects will receive up to 6 total HERCEPTIN® doses. After the last subject has received the last cycle of paclitaxel, data collection for safety and pharmacokinetic analysis will stop, and the study will close to protocol specified treatment. Study subjects may continue to receive the HERCEPTIN® +/− paclitaxel at the discretion of the investigator.

It is believed that the above treatment regimen will be effective in treating metastatic breast cancer, despite the infrequency with which HERCEPTIN® is administered to the patient.

While the particular aspects and embodiments of the invention as herein shown and disclosed in detail is fully capable of obtaining the objects and providing the advantages herein before stated, it is to be understood that it is merely illustrative of some of the presently preferred embodiments of the invention and that no limitations are intended to the details of methods and articles of manufacture shown other than as described in the appended claims. The disclosures of all citations in the specification are expressly incorporated herein by reference.

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<223> OTHER INFORMATION: salvage receptor binding epitope

<400> SEQUENCE: 6

His Gln Asn Ile Ser Asp Gly Lys
1 5

<210> SEQ ID NO 7
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: salvage receptor binding epitope

<400> SEQUENCE: 7

Val Ile Ser Ser His Leu Gly Gln
1 5

<210> SEQ ID NO 8
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val
1 5 10 15

Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
20 25 30

Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val
35 40 45

Ala Cys Ala His Tyr Lys Asp Pro Pro Phe Cys Val Ala Arg
50 55

<210> SEQ ID NO 9
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr
1 5 10 15

Cys Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr
20 25 30

Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
35 40 45

Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu
50 55 60

Gly Ala Cys Gln Pro
65

<210> SEQ ID NO 10
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<213> ORGANISM: Mus Musculus

-continued

<400> SEQUENCE: 10

Asp Thr Val Met Thr Gln Ser His Lys Ile Met Ser Thr Ser Val
1 5 10 15

Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Ser
20 25 30

Ile Gly Val Ala Trp Tyr Gln Gln Arg Pro Gly Gln Ser Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Asp
50 55 60

Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
65 70 75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
80 85 90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu
95 100 105

Ile Lys

<210> SEQ ID NO 11
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<213> ORGANISM: Mus musculus

<400> SEQUENCE: 11

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Thr Phe Thr
20 25 30

Asp Tyr Thr Met Asp Trp Val Lys Gln Ser His Gly Lys Ser Leu
35 40 45

Glu Trp Ile Gly Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
50 55 60

Asn Gln Arg Phe Lys Gly Lys Ala Ser Leu Thr Val Asp Arg Ser
65 70 75

Ser Arg Ile Val Tyr Met Glu Leu Arg Ser Leu Thr Phe Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
95 100 105

Phe Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
110 115

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<223> OTHER INFORMATION: humanized VL sequence

<400> SEQUENCE: 12

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1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser
20 25 30

Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
50 55 60

-continued

```
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys
```

<210> SEQ ID NO 13
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<223> OTHER INFORMATION: humanized VH sequence

<400> SEQUENCE: 13

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
                20                  25                  30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
               110                 115
```

<210> SEQ ID NO 14
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: VL consensus sequence

<400> SEQUENCE: 14

```
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  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
                20                  25                  30

Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys
```

-continued

<210> SEQ ID NO 15
<211> LENGTH: 119
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<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: VH consensus sequence

<400> SEQUENCE: 15

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                20                  25                  30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
                95                 100                 105

Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
               110                 115
```

The invention claimed is:

1. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising:

administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB2 antibody; and

administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose, wherein the subsequent doses are separated in time from each other by at least two weeks; and

further comprising administering an effective amount of a chemotherapeutic agent to the patient.

2. The method of claim **1**, wherein the initial dose is at least approximately 6 mg/kg.

3. The method of claim **2**, wherein the initial dose is at least approximately 8 mg/kg.

4. The method of claim **3**, wherein the initial dose is at least approximately 12 mg/kg.

5. The method of claim **1**, wherein the subsequent doses are separated in time from each other by at least three weeks.

6. The method of claim **1**, wherein the initial dose is administered by intravenous injection, and wherein at least one subsequent dose is administered by subcutaneous injection.

7. The method of claim **1**, wherein the initial dose is administered by intravenous injection, wherein at least two subsequent doses are administered, and wherein each subsequent dose is administered by a method selected from the group consisting of intravenous injection and subcutaneous injection.

8. The method of claim **1**, wherein the initial dose and at least one subsequent dose are administered by subcutaneous injection.

9. The method of claim **1**, wherein the initial dose is selected from the group consisting of approximately 6 mg/kg, 8 mg/kg, or 12 mg/kg, wherein the plurality of subsequent doses are at least approximately 2 mg/kg.

10. The method of claim **9**, wherein the plurality of subsequent doses are separated in time from each other by at least three weeks.

11. The method of claim **10**, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

12. The method of claim **10**, wherein the initial dose is approximately 12 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

13. The method of claim **9**, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 8 mg/kg.

14. The method of claim **9**, wherein the initial dose is approximately 8 mg/kg, wherein at least one subsequent dose is 8 mg/kg, and wherein administration of the initial dose and subsequent doses are separated in time by at least 2 weeks.

15. The method of claim **14**, wherein the initial dose and subsequent doses are separated in time by at least 3 weeks.

16. The method of claim **1**, wherein said cancer is selected from the group consisting of breast cancer, leukemia, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

17. The method of claim **16**, wherein said cancer is breast cancer.

18. The method of claim **17**, wherein said cancer is metastatic breast carcinoma.

19. The method of claim **1**, wherein said antibody binds to the extracellular domain of the ErbB2 receptor.

20. The method of claim **19**, wherein said antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

21. The method of claim **20**, wherein said antibody is a humanized 4D5 anti-ErbB2 antibody.

22. The method of claim **1**, wherein the chemotherapeutic agent is a taxoid.

23. The method of claim **22**, wherein said taxoid is paclitaxel or docetaxel.

24. The method of claim **1**, wherein the effective amount of the anti-ErbB2 antibody and the effective amount of the chemotherapeutic agent as a combination is lower than the sum of the effective amounts of said anti-ErbB2 antibody and said chemotherapeutic agent, when administered individually, as single agents.

25. The method of claim **1**, wherein the chemotherapeutic agent is an anthracycline.

26. The method of claim **25**, wherein the anthracycline is doxorubicin or epirubicin.

27. The method of claim **25**, wherein the method further comprises administration of a cardioprotectant.

28. The method of claim **1**, wherein efficacy is measured by determining the time to disease progression or the response rate.

29. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising: administering to the patient an initial dose of the antibody, wherein the initial dose is a plurality of doses, wherein each of the plurality of initial doses is at least approximately 1 mg/kg and is administered on at least 3 consecutive days, and administering to the patient at least one subsequent dose of the antibody, wherein at least one subsequent dose is at least approximately 6 mg/kg, and wherein administration of the last initial dose and the first subsequent and additional subsequent doses are separated in time by at least 3 weeks, and further comprising administering an effective amount of a chemotherapeutic agent to the patient.

30. A method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by two or more subsequent doses of the antibody, wherein the subsequent doses are separated from each other in time by at least about two weeks, and further comprising administering an effective amount of a chemotherapeutic agent to the patient.

31. The method of claim **30**, wherein the first dose and a first subsequent dose are separated from each other in time by at least about three weeks.

32. The method of claim **30**, wherein the first dose and subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

33. The method of claim **32**, wherein the first dose and subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

34. The method of claim **33**, wherein the first dose and subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

35. The method of claim **30**, wherein from about two to about ten subsequent doses of the antibody are administered to the patient.

36. The method of claim **30**, wherein the two or more subsequent doses are separated from each other in time by at least about three weeks.

37. The method of claim **30**, wherein the two or more subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

38. The method of claim **30**, wherein the two or more subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

39. The method of claim **30**, wherein the two or more subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

40. The method of claim **30**, wherein the chemotherapeutic agent is a taxoid.

* * * * *

EXHIBIT I

US006417335B1

(12) **United States Patent**
Basey et al.

(10) **Patent No.: US 6,417,335 B1**
(45) **Date of Patent: Jul. 9, 2002**

(54) **PROTEIN PURIFICATION**

(75) Inventors: **Carol D. Basey**, Winters; **Greg S. Blank**, Menlo Park, both of CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/680,148**

(22) Filed: **Oct. 3, 2000**

Related U.S. Application Data

(62) Division of application No. 09/304,465, filed on May 3, 1999.
(60) Provisional application No. 60/084,459, filed on May 6, 1998.

(51) **Int. Cl.**[7] **C07K 1/18**; C07K 16/00
(52) **U.S. Cl.** **530/387.1**; 530/387.3; 530/387.7; 530/388.85; 530/389.7; 530/416
(58) **Field of Search** 530/416, 387.1, 530/387.3, 387.7, 388.85, 389.7

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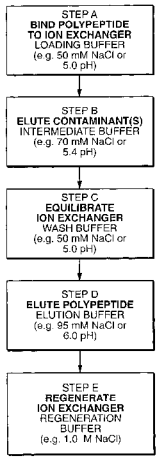
Primary Examiner—David Saunders
(74) *Attorney, Agent, or Firm*—Wendy M. Lee

(57) **ABSTRACT**

A method for purifying a polypeptide by ion exchange chromatography is described which involves changing the conductivity and/or pH of buffers in order to resolve a polypeptide of interest from one or more contaminants.

9 Claims, 7 Drawing Sheets



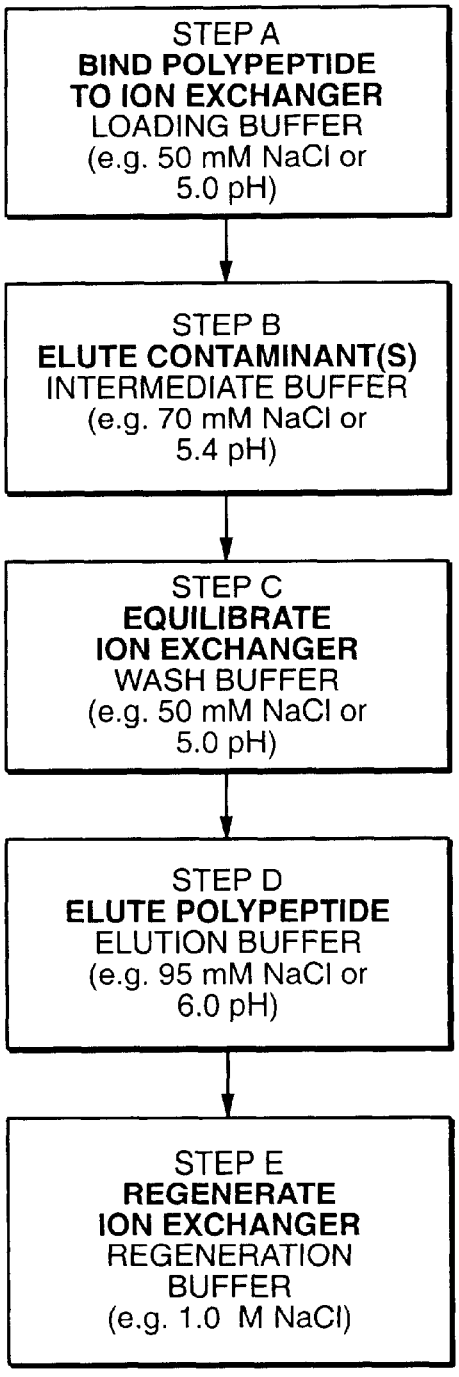

STEP A
BIND POLYPEPTIDE
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
5.4 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
6.0 pH)
STEP E
REGENERATE
ION EXCHANGER
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._1

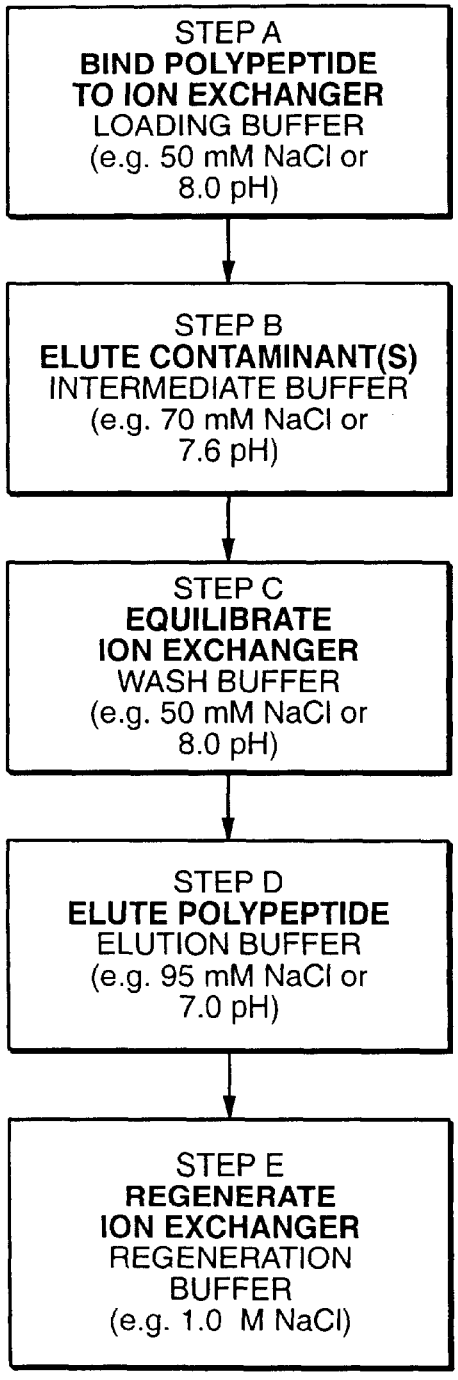

STEP A
BIND POLYPEPTIDE
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
7.6 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
7.0 pH)
STEP E
REGENERATE
ION EXCHANGER
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._2

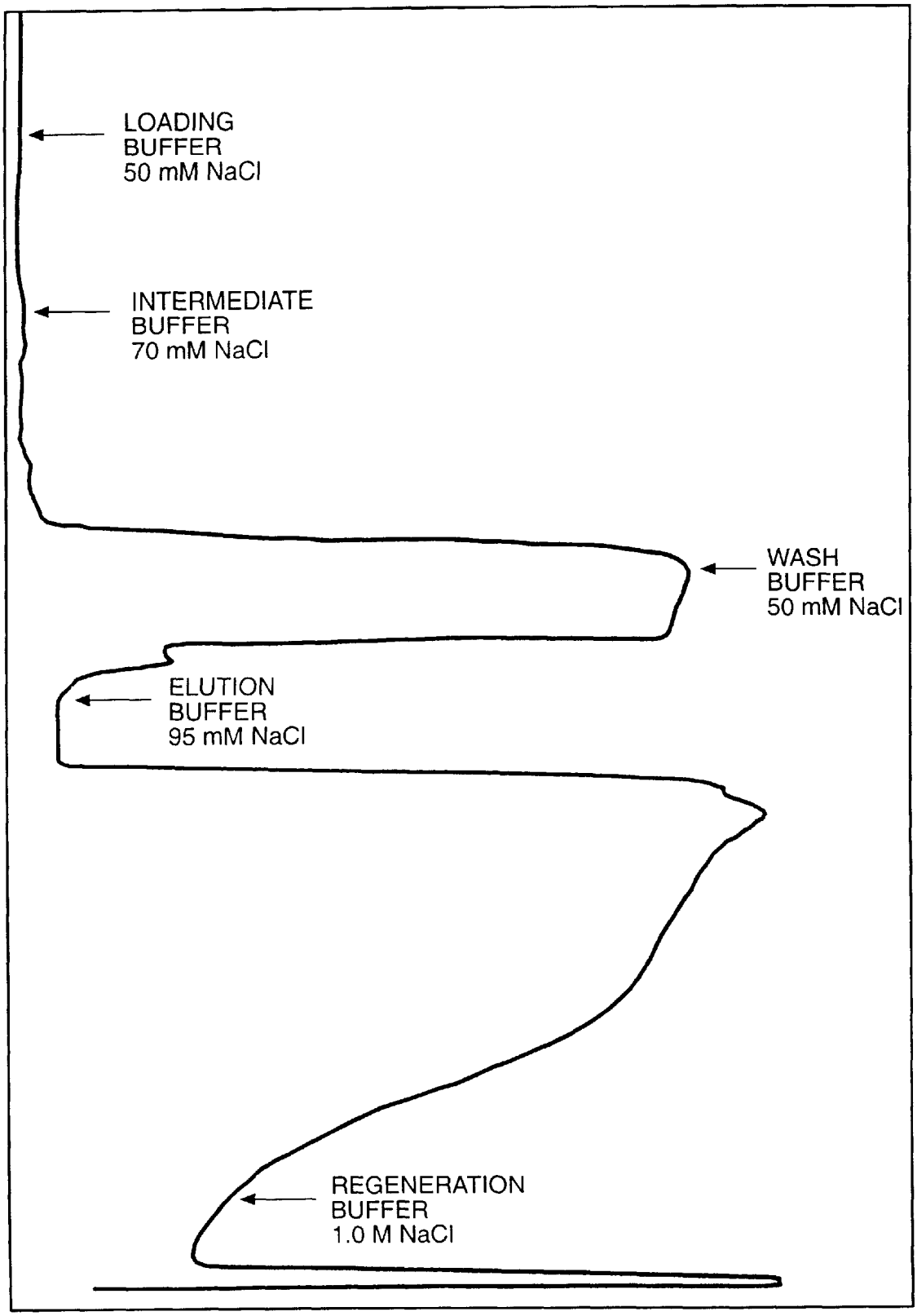

LOADING
BUFFER
50 mM NaCl
INTERMEDIATE
BUFFER
70 mM NaCl
WASH
BUFFER
50 mM NaCl
ELUTION
BUFFER
95 mM NaCl
REGENERATION
BUFFER
1.0 M NaCl

FIG._3

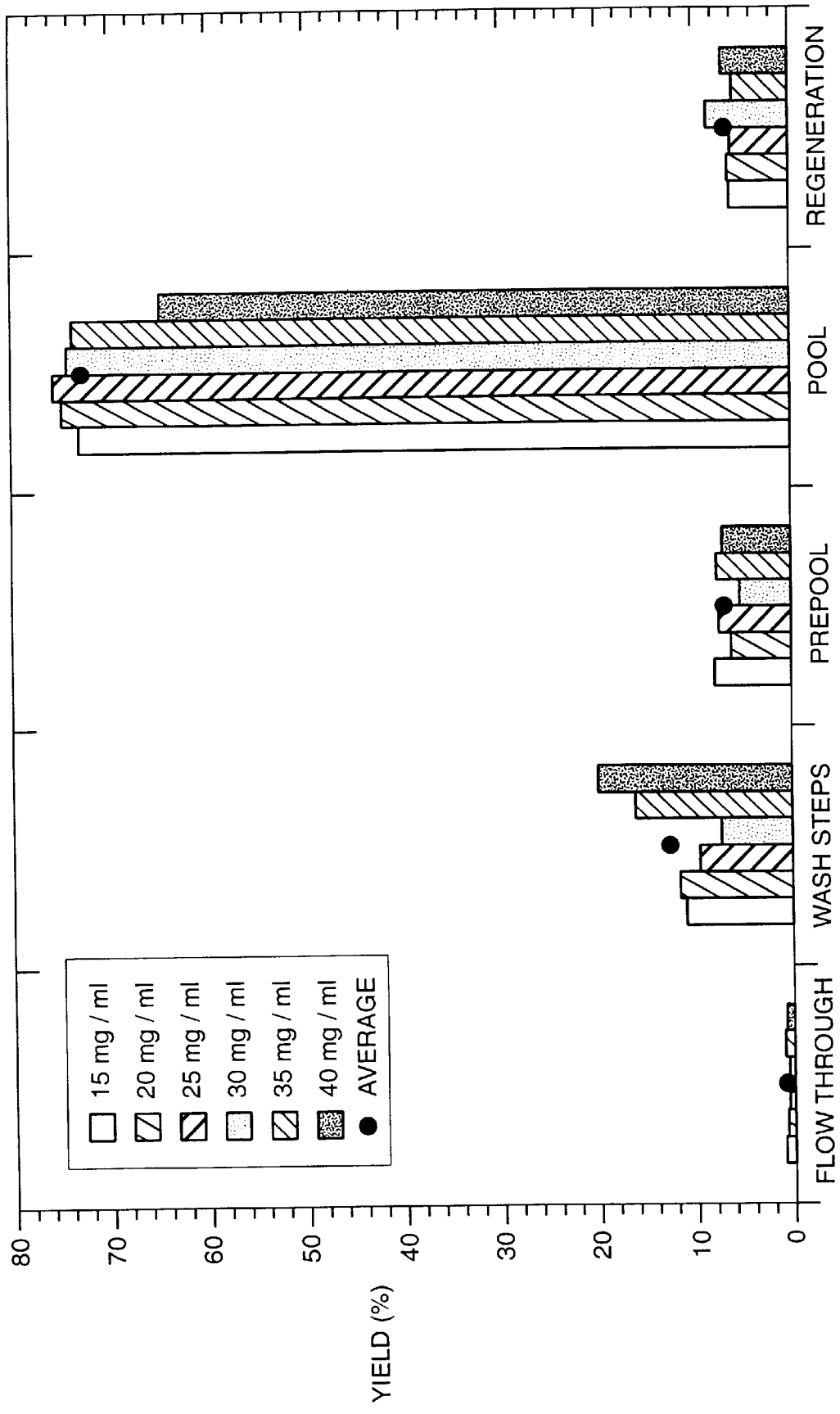

YIELD (%)
0
10
20
30
40
50
60
70
80
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
AVERAGE
FLOW THROUGH
WASH STEPS
PREPOOL
POOL
REGENERATION
CHROMATOGRAPHY FRACTION

FIG._4

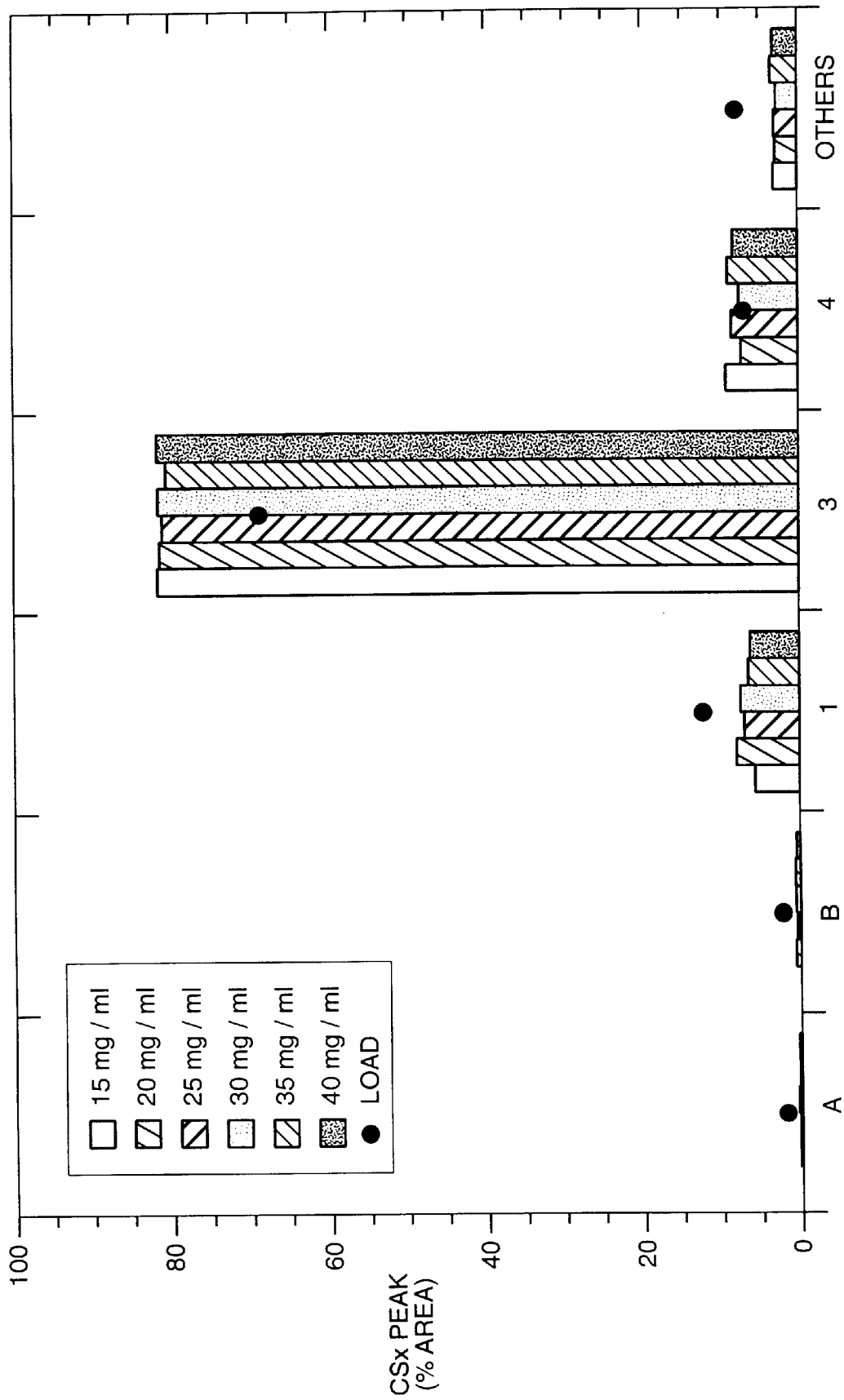

CSx PEAK (% AREA)
100
80
60
40
20
0
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
LOAD
A
B
1
3
4
OTHERS
CSx PEAK

FIG._5

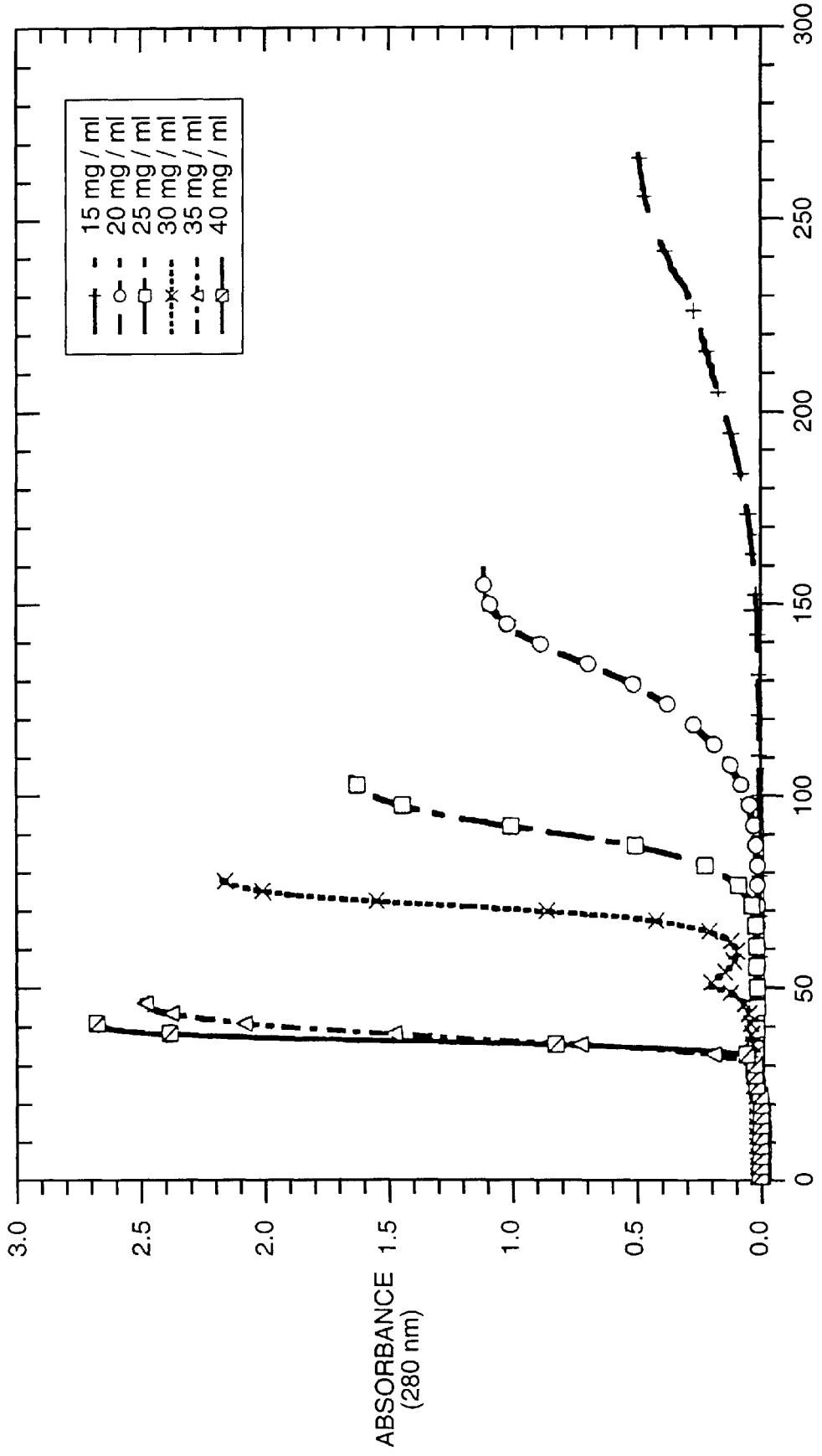

15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
ABSORBANCE (280 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
TIME (MINUTES)

FIG._6

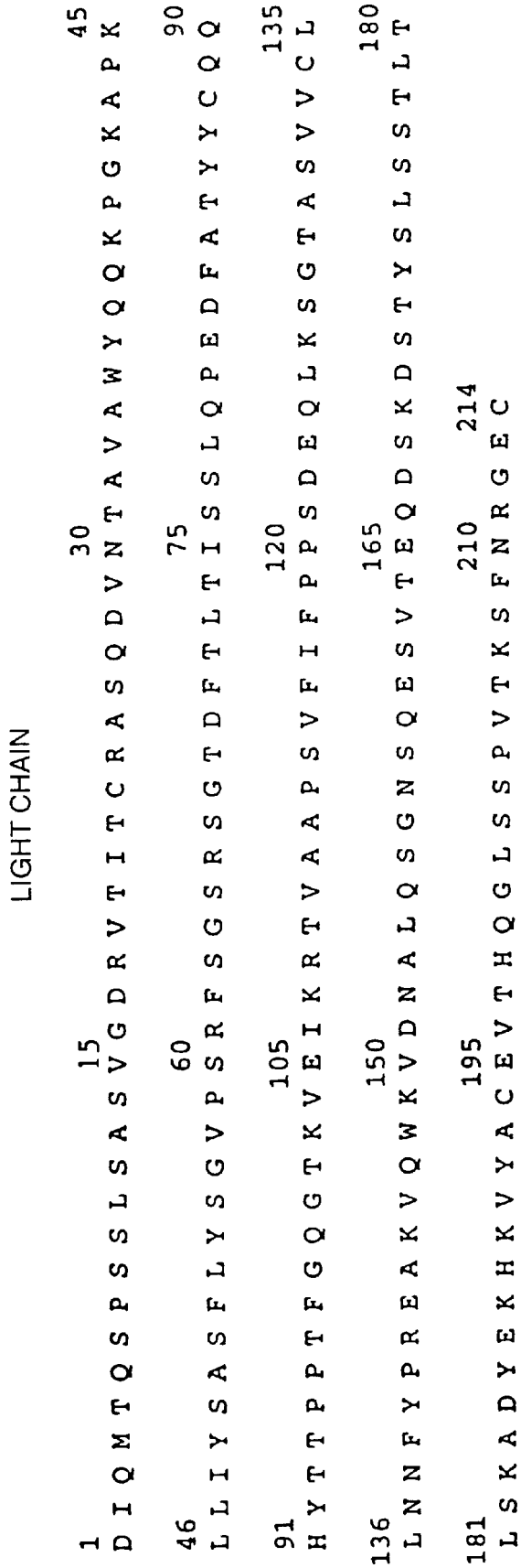

LIGHT CHAIN
1 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q D V N T A V A W Y Q Q K P G K A P K
46 L L I Y S A S F L Y S G V P S R F S G S R S G T D F T L T I S S L Q P E D F A T Y Y C Q Q
91 H Y T T P P T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L
136 L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T
181 L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C

FIG._7A

HEAVY CHAIN

1	E V Q L V E S G G G L V Q P G G S L R L S C A A S G F N I K D T Y I H W V R Q A P G K G L	45
46	E W V A R I Y P T N G Y T R Y A D S V K G R F T I S A D T S K N T A Y L Q M N S L R A E D	90
91	T A V Y Y C S R W G G D G F Y A M D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S	135
136	K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S	180
181	G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K	225
226	T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S	270
271	H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D	315
316	W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E	360
361	M T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G	405
406	S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G	449

FIG._7B

PROTEIN PURIFICATION

This is a divisional of application Ser. No. 09/304,465 filed May 3, 1999 which claims priority under 35 USC §119 to provisional application Ser. No. 60/084,459 filed May 6, 1998, both disclosures of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to protein purification. In particular, the invention relates to a method for purifying a polypeptide (e.g. an antibody) from a composition comprising the polypeptide and at least one contaminant using the method of ion exchange chromatography.

2. Description of Related Art

The large-scale, economic purification of proteins is increasingly an important problem for the biotechnology industry. Generally, proteins are produced by cell culture, using either mammalian or bacterial cell lines engineered to produce the protein of interest by insertion of a recombinant plasmid containing the gene for that protein. Since the cell lines used are living organisms, they must be fed with a complex growth medium, containing sugars, amino acids, and growth factors, usually supplied from preparations of animal serum. Separation of the desired protein from the mixture of compounds fed to the cells and from the by-products of the cells themselves to a purity sufficient for use as a human therapeutic poses a formidable challenge.

Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through".

Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins.

In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; i.e., the pH or conductivity is increased or decreased in a single direction.

SUMMARY OF THE INVENTION

The present invention provides an ion exchange chromatographic method wherein a polypeptide of interest is bound to the ion exchange material at an initial conductivity or pH and then the ion exchange material is washed with an intermediate buffer at a different conductivity or pH, or both. At a specific point following this intermediate wash, and contrary to ion exchange chromatography standard practice, the ion exchange material is washed with a wash buffer where the change in conductivity or pH, or both, from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity or pH, or both, achieved in the previous steps. Only after washing with the wash buffer, is the ion exchange material prepared for the polypeptide molecule of interest to be eluted by the application of the elution buffer having a conductivity or pH, or both, which differ from the conductivity or pH, or both, of the buffers used in previous steps.

This novel approach to ion exchange chromatography is particularly useful in situations where a product molecule must be separated from a very closely related contaminant molecule at full manufacturing scale, where both purity and high recovery of polypeptide product are desired.

Accordingly, the invention provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to an ion exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the ion exchange material with an intermediate buffer at a second conductivity and/or pH so as to elute the contaminant from the ion exchange material;

(c) washing the ion exchange material with a wash buffer which is at a third conductivity and/or pH, wherein the change in conductivity and/or pH from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity and/or pH from the loading buffer to the intermediate buffer; and

(d) washing the ion exchange material with an elution buffer at a fourth conductivity and/or pH so as to elute the polypeptide from the ion exchange material. The first conductivity and/or pH may be the same as the third conductivity and/or pH.

Where the ion exchange material comprises a cation exchange resin, the conductivity and/or pH of the intermediate buffer is/are preferably greater than the conductivity and/or pH of the loading buffer; the conductivity and/or pH of the wash buffer is/are preferably less than the conductivity and/or pH of the intermediate buffer; and the conductivity and/or pH of the elution buffer is/are preferably greater than the conductivity and/or pH of the intermediate buffer. Preferably, the conductivity and/or pH of the wash buffer is/are about the same as the conductivity and/or pH of the loading buffer.

Preferably elution of the contaminant and of the polypeptide is achieved by modifying the conductivity of the intermediate buffer and of the elution buffer, respectively, while keeping the pH of these buffers approximately the same.

The invention also provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to a cation exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the cation exchange material with an intermediate buffer at a second conductivity and/or pH which is greater than that of the loading buffer so as to elute the contaminant from the ion exchange material;

(c) washing the cation exchange material with a wash buffer which is at a third conductivity and/or pH which is less than that of the intermediate buffer; and

(d) washing the cation exchange material with an elution buffer at a fourth conductivity and/or pH which is greater than that of the intermediate buffer so as to elute the polypeptide from the ion exchange material.

In addition, the invention provides a method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from about 20 mg to about 35 mg of the antibody per mL of cation exchange resin and, optionally, further comprising eluting the antibody from the cation exchange resin. The method preferably further comprises an intermediate wash step for eluting one or more contaminants from the ion exchange resin. This intermediate wash step usually precedes the step of eluting the antibody.

The invention further provides a composition comprising a mixture of anti-HER2 antibody and one or more acidic variants thereof, wherein the amount of the acidic variant(s) in the composition is less than about 25% and preferably less than about 20%, e.g. in the range from about 1% to about 18%. Optionally, the composition further comprises a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flow diagram showing how one could perform cation exchange chromatography by altering conductivity (e.g. to the NaCl concentrations of Example 1 below) or by altering pH (e.g. to the pH values as shown in the flow diagram).

FIG. **2** is a flow diagram showing how one could perform anion exchange chromatography by altering conductivity (e.g. to the NaCl concentrations as depicted in the figure) or by altering pH (e.g. to the pH values as shown).

FIG. **3** is an absorbance trace from a cation exchange chromatography run of Example 1 at full manufacturing scale. Points at which the column is washed with the different buffers described herein are marked with arrows.

FIG. **4** depicts recombinant humanized anti-HER2 monoclonal antibody (rhuMAb HER2) recovered in each chromatography fraction (calculated as the percentage of the sum total of all fractions of the relevant chromatography). Flow through, wash steps, and prepool fractions are all effluent samples collected from the onset of load to the initiation of pooling. The pool fraction is the five column volume effluent sample of elution starting at the leading shoulder's inflection point. The regeneration fraction contains effluent captured from the end of pooling to the end of regeneration.

FIG. **5** shows the quality of rhuMAb HER2 in each cation exchange chromatography pool sample as evaluated by carboxy sulfon cation exchange high pressure liquid chromatography (CSx HPIEX). Peaks a, b, and 1 are deamidated forms of rhuMAb HER2. Peak 3 is nondeamidated rhuMAb HER2. Peak 4 is a combination of C-terminal Lysine containing and iso-aspartate variants of rhuMAb HER2.

FIG. **6** shows the absorbance (280 nm) profiles of the 0.025 M MES/0.070 M NaCl, pH 5.6 wash for each chromatography. The mass of rhuMAb HER2 applied to the cation exchange resin effects the peak's absorbance level at the apex as well as the amount of buffer required to reach the apex. Due to minor peaks which occur (as best seen in the 30 mg/mL load) in this wash, the apex is defined as absorbance levels of at least 0.5 absorbance units (AU).

FIGS. **7**A and **7**B show the amino acid sequences of humMAb4D5-8 light chain (SEQ ID NO:1) and humMAb4D5-8 heavy chain (SEQ ID NO:2), respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The "composition" to be purified herein comprises the polypeptide of interest and one or more contaminants. The composition may be "partially purified" (i.e. having been subjected to one or more purification steps, such as Protein A Chromatography as in Example 1 below) or may be obtained directly from a host cell or organism producing the polypeptide (e.g. the composition may comprise harvested cell culture fluid).

As used herein, "polypeptide" refers generally to peptides and proteins having more than about ten amino acids. Preferably, the polypeptide is a mammalian protein, examples of which include renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β1, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor

binding proteins (IGFBPs); CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides. Most preferred is a full length antibody that binds human HER2.

A "contaminant" is a material that is different from the desired polypeptide product. The contaminant may be a variant of the desired polypeptide (e.g. a deamidated variant or an amino-aspartate variant of the desired polypeptide) or another polypeptide, nucleic acid, endotoxin etc.

A "variant" or "amino acid sequence variant" of a starting polypeptide is a polypeptide that comprises an amino acid sequence different from that of the starting polypeptide. Generally, a variant will possess at least 80% sequence identity, preferably at least 90% sequence identity, more preferably at least 95% sequence identity, and most preferably at least 98% sequence identity with the native polypeptide. Percentage sequence identity is determined, for example, by the Fitch et al., *Proc. Natl. Acad. Sci. USA* 80:1382–1386 (1983), version of the algorithm described by Needleman et al., *J. Mol. Biol.* 48:443–453 (1970), after aligning the sequences to provide for maximum homology. Amino acid sequence variants of a polypeptide may be prepared by introducing appropriate nucleotide changes into DNA encoding the polypeptide, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites. Methods for generating amino acid sequence variants of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

An "acidic variant" is a variant of a polypeptide of interest which is more acidic (e.g. as determined by cation exchange chromatography) than the polypeptide of interest. An example of an acidic variant is a deamidated variant.

A "deamidated" variant of a polypeptide molecule is a polypeptide wherein one or more asparagine residue(s) of the original polypeptide have been converted to aspartate, i.e. the neutral amide side chain has been converted to a residue with an overall acidic character. Deamidated humMAb4D5 antibody from the Example below has Asn30 in CDR1 of either or both of the V_L regions thereof converted to aspartate. The term "deamidated human DNase" as used herein means human DNase that is deamidated at the asparagine residue that occurs at position 74 in the amino acid sequence of native mature human DNase (U.S. Pat. No. 5,279,823; expressly incorporated herein by reference).

The term "mixture" as used herein in reference to a composition comprising an anti-HER2 antibody, means the presence of both the desired anti-HER2 antibody and one or more acidic variants thereof. The acidic variants may comprise predominantly deamidated anti-HER2 antibody, with minor amounts of other acidic variant(s). It has been found, for example, that in preparations of anti-HER2 antibody obtained from recombinant expression, as much as about 25% of the anti-HER2 antibody is deamidated.

In preferred embodiments of the invention, the polypeptide is a recombinant polypeptide. A "recombinant polypeptide" is one which has been produced in a host cell which has been transformed or transfected with nucleic acid encoding the polypeptide, or produces the polypeptide as a result of homologous recombination. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing nucleic acid into a cell. Following transformation or transfection, the nucleic acid may integrate into the host cell genome, or may exist as an extrachromosomal element. The "host cell" includes a cell in vitro cell culture as well a cell within a host animal. Methods for recombinant production of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

The antibody herein is directed against an "antigen" of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those polypeptides discussed above. Preferred molecular targets for antibodies encompassed by the present invention include CD polypeptides such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and av/b3 integrin including either a or b subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; polypeptide C etc. Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations

which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). In a further embodiment, "monoclonal antibodies" can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552–554 (1990). Clackson et al., *Nature*, 352:624–628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779–783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265–2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255–258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24–34 (L1), 50–56 (L2) and 89–97 (L3) in the light chain variable domain and 31–35 (H1), 50–65 (H2) and 95–102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Polypeptides of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. residues 26–32 (L1), 50–52 (L2) and 91–96 (L3) in the light chain variable domain and 26–32 (H1), 53–55 (H2) and 96–101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901–917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The CDR and FR residues of the rhuMAb HER2 antibody of the example below (humAb4D5–8) are identified in Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107–117 (1992) and Brennan et al., *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163–167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185. "Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269–315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90:6444–6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata et al. *Polypeptide Eng.* 8(10):1057–1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

"Multispecific antibodies" have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-$p185^{HER2}$/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-$p185^{HER2}$, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell adhesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. FcγRI, or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-$p185^{HER2}$/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-RP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-D3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537–539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO 96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'—SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217–225 (1992) describe the production of a fully humanized bispecific antibody F(ab')2 molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547–1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444–15 6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites.

Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

The phrase "ion exchange material" refers to a solid phase which is negatively charged (i.e. a cation exchange resin) or positively charged (i.e. an anion exchange resin). The charge may be provided by attaching one or more charged ligands to the solid phase, e.g. by covalent linking. Alternatively, or in addition, the charge may be an inherent property of the solid phase (e.g. as is the case for silica, which has an overall negative charge).

By "solid phase" is meant a non-aqueous matrix to which one or more charged ligands can adhere. The solid phase may be a purification column, a discontinuous phase of discrete particles, a membrane, or filter etc. Examples of materials for forming the solid phase include polysaccharides (such as agarose and cellulose); and other mechanically stable matrices such as silica (e.g. controlled pore glass), poly(styrenedivinyl)benzene, polyacrylamide, ceramic particles and derivatives of any of the above.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, BAKERBOND ABX™, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from Pharmacia) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from Pharmacia).

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (Pharmacia).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in Buffers. *A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., Ed. Calbiochem Corporation (1975). In one

embodiment, the buffer has a pH in the range from about 5 to about 7 (e.g. as in Example 1 below). Examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more contaminants onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more contaminants) is/are bound to the ion exchange resin.

The "intermediate buffer" is used to elute one or more contaminants from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that the contaminant is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all contaminants and the polypeptide of interest from the ion exchange resin.

The term "conductivity" refers to the ability of an aqueous solution to conduct an electric current between two electrodes. In solution, the current flows by ion transport. Therefore, with an increasing amount of ions present in the aqueous solution, the solution will have a higher conductivity. The unit of measurement for conductivity is mmhos (mS/cm), and can be measured using a conductivity meter sold, e.g., by Orion. The conductivity of a solution may be altered by changing the concentration of ions therein. For example, the concentration of a buffering agent and/or concentration of a salt (e.g. NaCl or KCl) in the solution may be altered in order to achieve the desired conductivity. Preferably, the salt concentration of the various buffers is modified to achieve the desired conductivity as in the Example below.

By "purifying" a polypeptide from a composition comprising the polypeptide and one or more contaminants is meant increasing the degree of purity of the polypeptide in the composition by removing (completely or partially) at least one contaminant from the composition. A "purification step" may be part of an overall purification process resulting in a "homogeneous" y composition, which is used herein to refer to a composition comprising at least about 70% by weight of the polypeptide of interest, based on total weight of the composition, preferably at least about 80% by weight.

Unless indicated otherwise, the term "HER2" when used herein refers to human HER2 protein and "HER2" refers to human HER2 gene. The human HER2 gene and HER2 protein are described in Semba et al., *PNAS (USA)* 82:6497–6501 (1985) and Yamamoto et al. *Nature* 319:230–234 (1986) (Genebank accession number X03363), for example.

The term "humMAb4D5–8" when used herein refers to a humanized anti-HER2 antibody comprising the light chain amino acid sequence of SEQ ID NO:1 and the heavy chain amino acid sequence of SEQ ID NO:2 or amino acid sequence variants thereof which retain the ability to bind HER2 and inhibit growth of tumor cells which overexpress HER2 (see U.S. Pat. No. 5,677,171; expressly incorporated herein by reference).

The "pI" or "isoelectric point" of a polypeptide refer to the pH at which the polypeptide's positive charge balances its negative charge. pI can be calculated from the net charge of the amino acid residues of the polypeptide or can be determined by isoelectric focussing (e.g. using CSx chromatography as in the Example below).

By "binding" a molecule to an ion exchange material is meant exposing the molecule to the ion exchange material under appropriate conditions (pH/conductivity) such that the molecule is reversibly immobilized in or on the ion exchange material by virtue of ionic interactions between the molecule and a charged group or charged groups of the ion exchange material.

By "washing" the ion exchange material is meant passing an appropriate buffer through or over the ion exchange material.

To "elute" a molecule (e.g. polypeptide or contaminant) from an ion exchange material is meant to remove the molecule therefrom by altering the ionic strength of the buffer surrounding the ion exchange material such that the buffer competes with the molecule for the charged sites on the ion exchange material.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with the polypeptide purified as described herein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the polypeptide. The label may be itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL™, Bristol-Myers Squibb Oncology, Princeton, N.J.), and doxetaxel, toxotere, methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

MODES FOR CARRYING OUT THE INVENTION

The invention herein provides a method for purifying a polypeptide from a composition (e.g. an aqueous solution) comprising the polypeptide and one or more contaminants. The composition is generally one resulting from the recombinant production of the polypeptide, but may be that resulting from production of the polypeptide by peptide synthesis (or other synthetic means) or the polypeptide may be purified from a native source of the polypeptide. Preferably the polypeptide is an antibody, e.g. one which binds the HER2 antigen.

For recombinant production of the polypeptide, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide is readily isolated and sequenced using conventional procedures (e.g., where the polypeptide is an antibody by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (e.g. as described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., *E. coli*, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., *Salmonella typhimurium*, Serratia, e.g., *Serratia marcescans*, and Shigella, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as *P. aeruginosa*, and Streptomyces. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; Kluyveromyces hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; yarrowia (EP 402,226); *Pichia pastoris* (EP 183,070); Candida; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; Schwanniomyces such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243–251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44–68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (e.g. resulting from homogenization), is removed, for example, by centrifuga-

tion or ultrafiltration. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

The polypeptide is then subjected to one or more purification steps, including the ion exchange chromatography method as claimed herein. Examples of additional purification procedures which may be performed prior to, during, or following the ion exchange chromatography method include fractionation on a hydrophobic interaction chromatography (e.g. on phenyl sepharose), ethanol precipitation, isoelectric focusing, Reverse Phase HPLC, chromatography on silica, chromatography on HEPARIN SEPHAROSE™, further anion exchange chromatography and/or further cation exchange chromatography, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography (e.g. using protein A, protein G, an antibody, a specific substrate, ligand or antigen as the capture reagent).

Ion exchange chromatography is performed as claimed herein. A decision is first made as to whether an anion or cation exchange resin is to be employed. In general, a cation exchange resin may be used for polypeptides with pIs greater than about 7 and an anion exchange resin may be used for polypeptides with pIs less than about 7.

The anion or cation exchange resin is prepared according to known methods. Usually, an equilibration buffer is passed through the ion exchange resin prior to loading the composition comprising the polypeptide and one or more contaminants onto the resin. Conveniently, the equilibration buffer is the same as the loading buffer, but this is not required.

The various buffers used for the chromatography depend, for example, on whether a cation or anion exchange resin is employed. This is shown more clearly in the flow diagrams of FIGS. **1** and **2**.

With particular reference to FIG. **1**, which shows exemplary steps to be performed where a cation exchange resin is used, the pH and/or conductivity of each buffer is/are increased relative to the preceding buffer, except for the wash buffer where the conductivity and/or pH is/are less than the conductivity and/or pH of the preceding intermediate buffer. The aqueous solution comprising the polypeptide of interest and contaminant(s) is loaded onto the cation exchange resin using the loading buffer that is at a pH and/or conductivity such that the polypeptide and the contaminant bind to the cation exchange resin. As in the Example below, the loading buffer may be at a first low conductivity (e.g. from about 5.2 to about 6.6 mmhos). An exemplary pH for the loading buffer may be about 5.0 (see FIG. **1**). From about 20 mg/mL to about 35 mg/mL of the polypeptide (e.g. of a full length antibody) may, for example, be loaded on the ion exchange resin.

The cation exchange resin is then washed with an intermediate buffer which is at a second conductivity and/or pH so as to essentially elute the contaminant, but not a substantial amount of the polypeptide of interest. This may be achieved by increasing the conductivity or pH, or both, of the intermediate buffer. The change from loading buffer to intermediate buffer may be step-wise or gradual as desired. In the Example herein, the intermediate buffer had a greater conductivity than that of the loading buffer (i.e. the intermediate buffer's conductivity was in the range from about 7.3 to about 8.4 mmhos). Alternatively, as shown in FIG. **1**, the pH of the intermediate buffer may exceed that of the loading buffer in this embodiment of the invention, where a cation exchange resin is used. For example, the intermediate buffer may have a pH of about 5.4.

Following washing with the intermediate buffer, the cation exchange resin is washed or re-equilibrated with the wash buffer which has a conductivity or pH, or both, which is/are less than that of the intermediate buffer (i.e. the conductivity, or pH, or both, is/are changed in an opposite, i.e. reverse, direction to the preceding step, unlike ion exchange chromatography steps in the literature). In the Example below, the wash buffer had about the same conductivity as the loading buffer (i.e. in the range from about 5.2 to about 6.6 mmhos) and its conductivity was, therefore, less than that of the intermediate buffer. In another embodiment, one may reduce the conductivity of the wash buffer to a conductivity that is less than, or greater than, that of the loading buffer, provided the conductivity of the wash buffer is less than that of the intermediate buffer. In another embodiment, the pH of the wash buffer may be less than the pH of the intermediate buffer (e.g. the pH of the wash buffer may about 5.0). The change in conductivity and/or pH of the wash buffer compared to the intermediate buffer may be achieved by step-wise or gradual change of either or both of these parameters.

After the wash step of the preceding paragraph, the cation exchange resin is prepared for elution of the desired polypeptide molecule therefrom. This is achieved using an elution buffer that has a pH and/or conductivity such that the desired polypeptide no longer binds to the cation exchange resin and therefore is eluted therefrom. The pH and/or conductivity of the elution buffer generally exceed(s) the pH and/or conductivity of the loading buffer, the intermediate buffer and the wash buffer used in the previous steps. In the Example below, the conductivity of the elution buffer was in the range from about 10.0 to about 11.0 mmhos. Alternatively, or in addition, the pH of the elution buffer may be increased relative to the wash buffer and to the intermediate buffer (for example, the pH of the elution buffer may about 6.0). The change in conductivity and/or pH may be step-wise or gradual, as desired. Hence, the desired polypeptide is retrieved from the cation exchange resin at this stage in the method.

In an alternative embodiment, the ion exchange material comprises an anion exchange resin. This embodiment of the invention is depicted in FIG. **2** herein. As illustrated in this figure, the changes in conductivity are generally as described above with respect to a cation exchange resin. However, the direction of change in pH is different for an anion exchange resin. For example, if elution of the contaminant(s) and polypeptide are to be achieved by altering pH, the loading buffer has a first pH and the pH is decreased in the intermediate buffer so as to elute the contaminant or contaminants. In the third step, the column is washed/re-equilibrated with the wash buffer and the change in conductivity or pH, or both, is in the opposite direction to that of the previous step. Hence, the pH may be

increased in the wash buffer, compared to the intermediate buffer. Following this step, the polypeptide of interest is eluted from the anion exchange resin using an elution buffer at a fourth conductivity and/or pH. If pH is altered, it will normally be less than the pH of the loading buffer, the intermediate buffer and the wash buffer. The change in pH and/or conductivity in progressive buffers can, as explained above, be step-wise or gradual.

In the preferred embodiment of the invention, a single parameter (i.e. either conductivity or pH) is changed to achieve elution of both the polypeptide and contaminant, while the other parameter (i.e. pH or conductivity, respectively) remains about constant. For example, while the conductivity of the various buffers (loading buffer, intermediate buffer, wash buffer and/or elution buffer) may differ, the pH's thereof may be essentially the same.

In an optional embodiment of the invention, the ion exchange resin is regenerated with a regeneration buffer after elution of the polypeptide, such that the column can be re-used. Generally, the conductivity and/or pH of the regeneration buffer is/are such that substantially all contaminants and the polypeptide of interest are eluted from the ion exchange resin. Generally, the regeneration buffer has a very high conductivity for eluting contaminants and polypeptide from the ion exchange resin.

The method herein is particularly useful for resolving a polypeptide molecule of interest from at least one contaminant, where the contaminant and polypeptide molecule of interest differ only slightly in ionic charge. For example, the pIs of the polypeptide and contaminant may be only "slightly different", for example they may differ by only about 0.05 to about 0.2 pI units. In the Example below, this method-could be used to resolve an anti-HER2 antibody having a pI of 8.87, from a singly-deamidated variant thereof having a pI of 8.79. Alternatively, the method may be used to resolve a deamidated DNase, for example, from nondeamidated DNase. In another embodiment, the method may be used. to resolve a polypeptide from a glycosylation variant thereof, e.g. for resolving a variant of a polypeptide having. a different distribution of sialic acid compared to the nonvariant polypeptide.

The polypeptide preparation obtained according to the ion exchange chromatography method herein may be subjected to additional purification steps, if necessary. Exemplary further purification steps have been discussed above.

Optionally, the polypeptide is conjugated to one or more heterologous molecules as desired. The heterologous molecule may, for example, be one which increases the serum half-life of the polypeptide (e.g. polyethylene glycol, PEG), or it may be a label (e.g. an enzyme, fluorescent label and/or radionuclide) or a cytotoxic molecule (e.g. a toxin, chemotherapeutic drug, or radioactive isotope etc).

A therapeutic formulation comprising the polypeptide, optionally conjugated with a heterologous molecule, may be prepared by mixing the polypeptide having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. "Pharmaceutically acceptable" carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalqse or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). The humMAb4D5–8 antibody of particular interest herein may be prepared as a lyophilized formulation, e.g. as described in WO 97/04801; expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. For example, for an anti-HER2 antibody a chemotherapeutic agent, such as a taxoid or tamoxifen, may be added to the formulation.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulation to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide variant, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly (vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid.

The polypeptide purified as disclosed herein or the composition comprising the polypeptide and a pharmaceutically acceptable carrier is then used for various diagnostic, thera-

peutic or other uses known for such polypeptides and compositions. For example, the polypeptide may be used to treat a disorder in a mammal by administering a therapeutically effective amount of the polypeptide to the mammal.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1

Full length human-IgG rhuMAb HER2 (humAb4D5–8 in Carter et al. *Proc. Natl. Acad. Sci.* 89: 4285–4289 (1992) comprising the light chain amino acid sequence of SEQ ID NO:1 and heavy chain amino acid sequence of SEQ ID NO:2) was produced recombinantly in CHO cells. Following protein production and secretion to the cell culture medium, the CHO cells were separated from the cell culture medium by tangential flow filtration (PROSTACK™). Protein A chromatography was then performed by applying the Harvested Cell Culture Fluid (HCCF) from the CHO cells directly to an equilibrated PROSEP A™ column (Bioprocessing, Ltd).

Following Protein A chromatography, cation exchange chromatography was performed using a sulphopropyl (SP)-SEPHAROSE FAST FLOW™ (SPSFF) column (Pharmacia) to further separate the desired anti-HER2 antibody molecule. The chromatography operation was performed in bind and elute mode.

The SPSFF column was prepared for load by sequential washes with regeneration buffer (0.025 M MES/1.0 M NaCl, pH 5.6) followed by equilibration buffer (0.025 M MES/50 mM NaCl, pH 5.6). The column was then loaded with Protein A pool adjusted to a pH of 5.60±0.05 and a conductivity of 5.8±0.2 mmhos. Prior to elution, the column was washed in three steps: (1) loading buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1 column volume; (2) intermediate buffer (0.025 M MES/70 mM NaCl, pH 5.6) until an apex of a 280 nm peak was reached; and (3) wash buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1.2 column volumes. rhuMAb HER2 was then eluted from the column with elution buffer (0.025 M MES/95 mM NaCl, pH 5.6). The elution 280 nm profile has a shoulder on the leading edge (FIG. **3**). At the inflection point of this shoulder, pooling starts and continues for an additional 5 column volumes. The column was. then regenerated with regeneration buffer (0.025 M MES/1.0 M NaCl, pH 5.6).

MATERIALS AND METHODS

Column and Load Preparation: A reduced-scale SPSFF column was packed. The dimensions were: 27.0 mL volume, 1.0 cm diameter and 34.5 cm bed height. The pH of an aliquot of Protein A pool was titered to 5.6 with 1.5 M Tris base. The conductivity of the pool was reduced by the addition of an equal volume of sterile water for injection (SWFI).

Chromatography: The chromatography runs for this study were performed with Pharmacia's UNICORN™ FPLC system. The equilibration, load, and initial wash steps were performed at a linear flow rate of 200 cm/h. All chromatography steps were performed at a linear flow rate of 100 cm/h.

The sequence of chromatography steps are defined in Table 1. A total of six chromatography runs were performed with load densities of 15, 20, 25, 30, 35, and 40 mg of rhuMAb HER2 per mL of SPSFF resin.

TABLE 1

Chromatography Steps[1]

Chromatography Step	Buffer	Approximate Endpoint
Equilibration: Part 1	0.025 M MES/1.0 M NaCl, pH 5.6	2 CV[2]
Equilibration: Part 2	0.025 M MES/0.05 M NaCl, pH 5.6	pH: 5.6 ± 0.1 Cond.: 5.8 ± 0.2 mmhos
Load	Adjusted Protein A Pool	As Required
Wash 1	0.025 M MES/0.05 M NaCl, pH 5.6	1.5 CV
Wash 2	0.025 M MES/0.07 M NaCl, pH 5.6	Apex of Peak
Wash 3	0.025 M MES/0.05 M NaCl, pH 5.6	2 CV
Elution: Prepool	0.025 M MES/0.095 M NaCl, pH 5.6	To Leading Shoulder's Inflection Point (~1.2 CV)
Elution: Pool	0.025 M MES/0.095 M NaCl, pH 5.6	5 CV
Regeneration	0.025 M MES/1.0 M NaCl, pH 5.6	2 CV

[1]The equilibration of the resin was performed in manual mode; the remaining steps were executed from a Pharmacia Unicom Program.
[2]CV = column volume(s).

Total Protein: The protein concentration of each chromatography fraction (flow through, wash steps, elution prepool, elution pool, and regeneration) was determined by spectrophometric scans of each sample. The results were used to calculate product recovery yields. The extinction coefficient for rhuMAb HER2 is 1.45. Calculations used to derive the results (FIG. **4**) are:

$$\text{Protein Concentration (mg/mL)} = \frac{280\text{ nm}}{1.45} \times \text{Dilution Factor}$$

Protein Mass (mg) in Each Fraction=Protein Concentration (mg/mL)×Fraction Volume (mL)

$$\text{Yield (\%)} = \frac{\text{Fraction Mass (mg)}}{\text{Total Mass (mg)}} \times 100$$

Determination of rhuMAb HER2 Antibody Variants (CSx HPIEX): The rhuMAb HER2 SPSFF chromatography column resolves antibody variants. Fractions from each of the study chromatographies were tested for the relative amount of variant antibody by CSx HPIEX chromatography. A BAKERBOND WIDE-PORE™ CSx HPIEX column (4.6× 250 mm) was run at 1 mL/min at 55° C. The mobile phase was formed from a tertiary gradient (Table 2).

TABLE 2

Gradient Scheme

Time (min)	% A	% B	% C
0-Initial Conditions	49	1	50
10.0	40	10	50
50.0	33	17	50

TABLE 2-continued

Gradient Scheme			
Time (min)	% A	% B	% C
50.2	49	1	50
70.0	49	1	50

The column is run at 1 mL/min at 55° C.

The A buffer was 0.025 M MES, pH 5.9; the B buffer was 1 M Ammonium Acetate, pH 7.0; and the C solution was sterile water for injection. The column was equilibrated with the gradient's initial conditions (49% A; 1% B; and 50% C) and 200 μl of sample, diluted with SWFI and containing<300 μg protein, was injected. Each resulting chromatogram was integrated to determine the percent area of each peak for each fraction (Table 3 and FIG. **5**).

TABLE 3

CSx HPIEX analysis of rhuMAb HER2	
CSx Peak	rhuMAb HER2 Variant
a & b	Light Chain: Asn → Asp^{30} deamidation and Other unidentifiable variation by tryptic map
1	Light Chain: Asn → Asp^{30} deamidation
3	Fully Processed Antibody
4	Heavy Chain: Asp → $Iso\text{-}Asp^{102}$ and/or Heavy Chain: An Additional Lys^{450}
Others	Heavy Chain: Asp → Succinimide and/or Multiple permulations found in Peaks 1 and 4

Chromatograms Compared: The absorbance data (AU 280 nm) from each chromatography file was exported from Unicorn in ASCII format. The data from the 0.025 M MES/0.07 M NaCl, pH 5.6 wash was translated into Excel format and copied into KALEIDAGRAPH™. Using KALEIDAGRAPH™, the wash profiles were overlaid (FIG. **6**) and compared to each other.

RESULTS AND DISCUSSION

Deamidated and other acidic variants of rhuMAb HER2 were produced when the antibody was made by recombinant DNA technology (see e.g., CSx peaks a, b and 1 in FIG. **5**). The deamidated and other acidic variants constituted about 25% (calculated as area under the integrated curve or profile obtained by CSx chromatography) of the composition obtained from the initial Protein A chromatography step. It was discovered that the ion exchange method described herein could be used to substantially reduce the amount of deamidated and other acidic variants in the anti-HER2 composition, i.e. to about 13% or less (i.e. the amount of acidic variants in the preparation subjected to cation exchange chromatography as described herein was decreased by about 50% or more).

An absorbance trace from a cation exchange column run performed as described above is shown in FIG. **3**. This method resolved a deamidated variant of anti-HER2 antibody that differed only slightly from nondeamidated anti-HER2 antibody. The increase in conductivity from the initial conditions to the intermediate wash began to elute the deamidated anti-HER2 antibody. However, continued washing at this conductivity was found to elute nondeamidated anti-HER2 antibody, resulting in a loss of product. Proceeding directly from the intermediate buffer to the elution buffer was observed to result in either an unacceptably low removal of deamidated anti-HER2 antibody from the product if pooling began early or unacceptably low yields of anti-HER2 antibody product if pooling was delayed until the deamidated anti-HER2 antibody was reduced. It was discovered that by going back to lower conductivity as used initially, the elution of deamidated anti-HER2 antibody continued, without significant anti-HER2 antibody product elution.

The effect of rhuMAb HER2 load on (a) buffer requirements, (b) product recovery in the pool, and (c) product quality in the pool was evaluated.

At load densities of 15 mg/mL up to 35 mg/mL, the product yield in the elution pool is approximately 75%. For the load density of 40 mg/mL, the product yield in the pool dropped to 65% (FIG. **4**). This reduced recovery in the pool is largely attributed to an increased antibody in the two wash steps (at 70 mM NaCl and 50 mM NaCl, respectively).

The quality of rhuMAb HER2 in all the elution pools is equivalent as determined by CSx HPIEX analysis (FIG. **5**). Compared to the load material; there is an enrichment of the nondeamidated antibody (Peak 3), no change in the amount $Iso\text{-}Asp^{102}$ or Lys^{450} antibody (Peak 4), and a reduction of the amount of Asp^{30} deamidated antibody (Peaks a, b, 1 and others).

The quality of rhuMAb HER2 in these cation pools is improved through the intermediate wash step. As the mass of rhuMAb HER2 bound to the resin increases, the intermediate buffer volume consumption needed to reach the apex of the 280 nm peak decreases. The buffer volume required for a 40 mg/mL load density is approximately 2.5 column volumes. The buffer volume required for a 15 mg/mL load density is approximately 15 column volumes. The exact increase of buffer requirement is not linear with the 5 mg/mL incremental changes between these two extremes. The greatest increase is seen between the load densities of 20 mg/mL and 15 mg/mL. Here the requirement doubles from 7.5 column volumes to the previously mentioned 15 column volumes of buffer. If the apex of the 70 mM NaCl wash peak is reached, however, the product quality is equivalent for any of load densities examined.

This study determined how much rhuMAb HER2 can be loaded onto the SPSFF resin. Between the ranges of 15 to 40 mg of antibody per mL of resin, there is no difference in the quality of rhuMAb HER2 recovered in the elution pool. The quantity of rhuMAb HER2 recovered, however, is reduced by approximately 10% when the resin is loaded with greater than 35 mg/mL. For consistent yields it is recommended that 35 mg/mL be set as the maximum load for manufacture of rhuMAb HER2. Furthermore, due to the substantial increase in the 70 mM NaCl wash volume requirement between the 20 and 15 mg/mL; it is recommended that 20 mg/mL be set as the minimal load for manufacture of rhuMAb HER2.

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Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                 20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys
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<210> SEQ ID NO 2
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<212> TYPE: PRT
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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                 20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                 50                  55                  60
```

-continued

```
Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                 65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                 95                 100                 105

Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                 120

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser
                125                 130                 135

Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys
                140                 145                 150

Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
                155                 160                 165

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
                170                 175                 180

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
                185                 190                 195

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
                200                 205                 210

Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
                215                 220                 225

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                230                 235                 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
                260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
                275                 280                 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
                290                 295                 300

Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
                305                 310                 315

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
                320                 325                 330

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
                335                 340                 345

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
                350                 355                 360

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
                365                 370                 375

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
                380                 385                 390

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
                395                 400                 405

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
                410                 415                 420

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
                425                 430                 435

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                440                 445
```

What is claimed is:

1. A method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises:

(a) loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from about 20 mg to about 35 mg of the antibody per mL of cation exchange resin; and

(b) eluting the antibody from the cation exchange resin.

2. The method of claim **1** wherein the cation exchange resin comprises sulphopropyl immobilized on agarose.

3. The method of claim **1** further comprising eluting the contaminant from the cation exchange resin in an intermediate wash step prior to eluting the antibody from the cation exchange resin.

4. A method for purifying an anti-HER2 antibody from a composition comprising the antibody and a contaminant, which method comprises:

(a) loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from about 20 mg to about 35 mg of the antibody per mL of cation exchange resin; and

(b) eluting the antibody from the cation exchange resin.

5. The method of claim **4** wherein the antibody is humMAb4D5–8.

6. The method of claim **4** further comprising eluting the contaminant from the cation exchange resin in an intermediate wash step prior to eluting the antibody from the cation exchange resin.

7. A method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises:

(a) loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from 20 mg to 35 mg of the antibody per mL of cation exchange resin;

(b) eluting the contaminant from the cation exchange resin in an intermediate wash step; and

(c) eluting the antibody from the cation exchange resin.

8. The method of claim **7** wherein the antibody is an anti-HER2 antibody.

9. The method of claim **8** wherein the antibody is humMAb4D5–8.

* * * * *

EXHIBIT J

US006489447B1

(12) **United States Patent**
Basey et al.

(10) **Patent No.: US 6,489,447 B1**
(45) **Date of Patent: Dec. 3, 2002**

(54) **PROTEIN PURIFICATION**

(75) Inventors: **Carol D. Basey**, Winters, CA (US); **Greg S. Blank**, Menlo Park, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/304,465**

(22) Filed: **May 3, 1999**

Related U.S. Application Data

(60) Provisional application No. 60/084,459, filed on May 6, 1998.

(51) **Int. Cl.**[7] **C07K 1/18**; C07K 16/00

(52) **U.S. Cl.** **530/387.1**; 530/387.3; 530/387.7; 530/388.85; 530/389.7; 530/402; 530/416

(58) **Field of Search** 530/416, 387.1, 530/387.3, 387.7, 388.85, 389.7, 402

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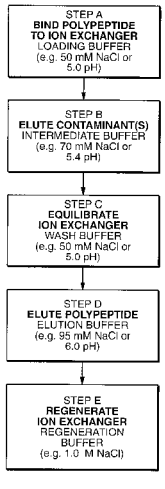
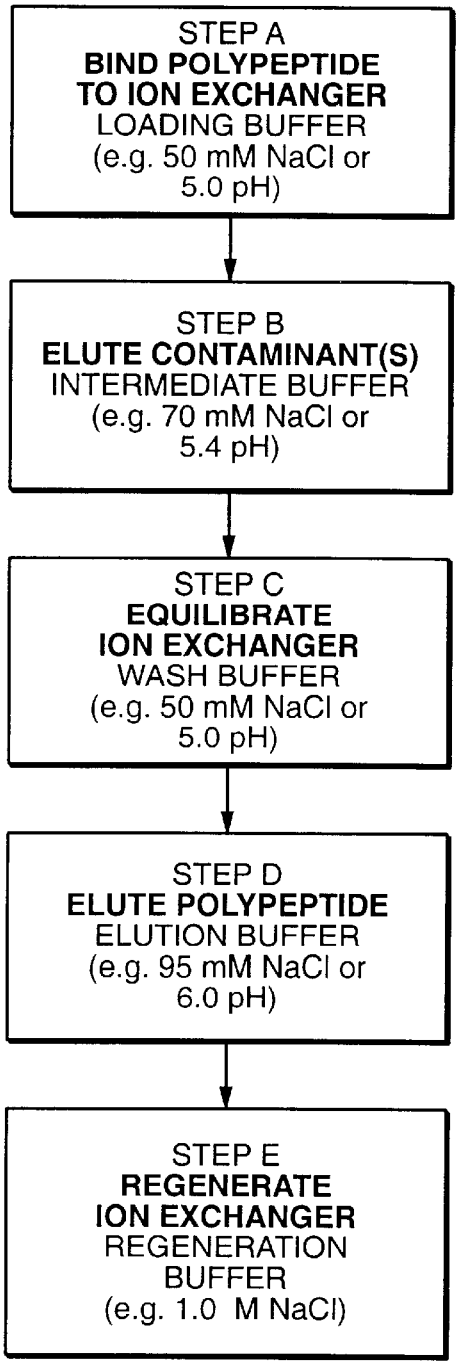
Primary Examiner—David Saunders
(74) *Attorney, Agent, or Firm*—Wendy M. Lee

(57) **ABSTRACT**

A method for purifying a polypeptide by ion exchange chromatography is described which involves changing the conductivity and/or pH of buffers in order to resolve a polypeptide of interest from one or more contaminants.

22 Claims, 7 Drawing Sheets




STEP A
BIND POLYPEPTIDE
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
5.4 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
6.0 pH)
STEP E
REGENERATE
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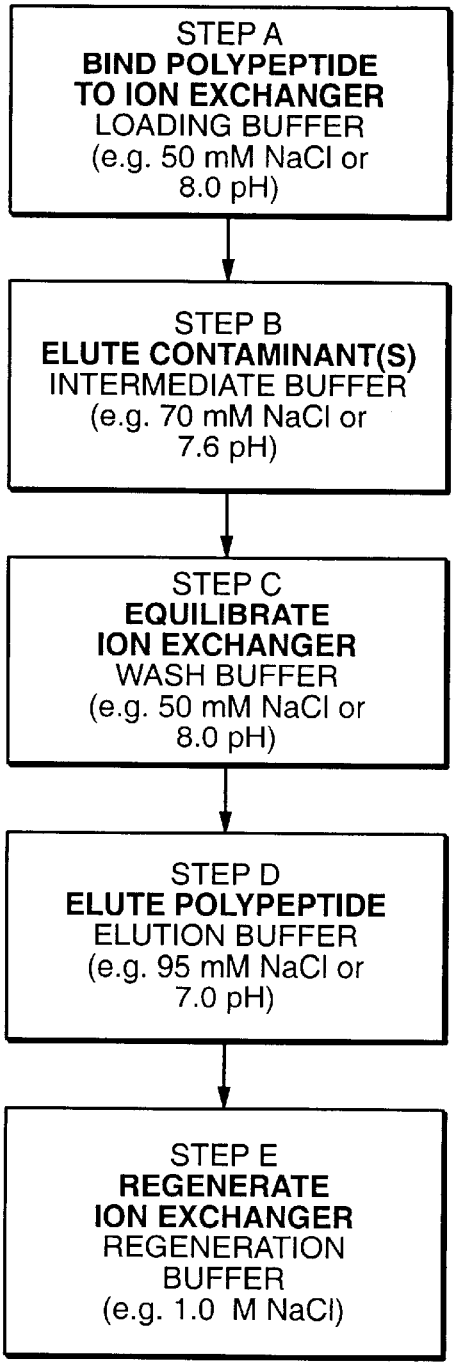
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._1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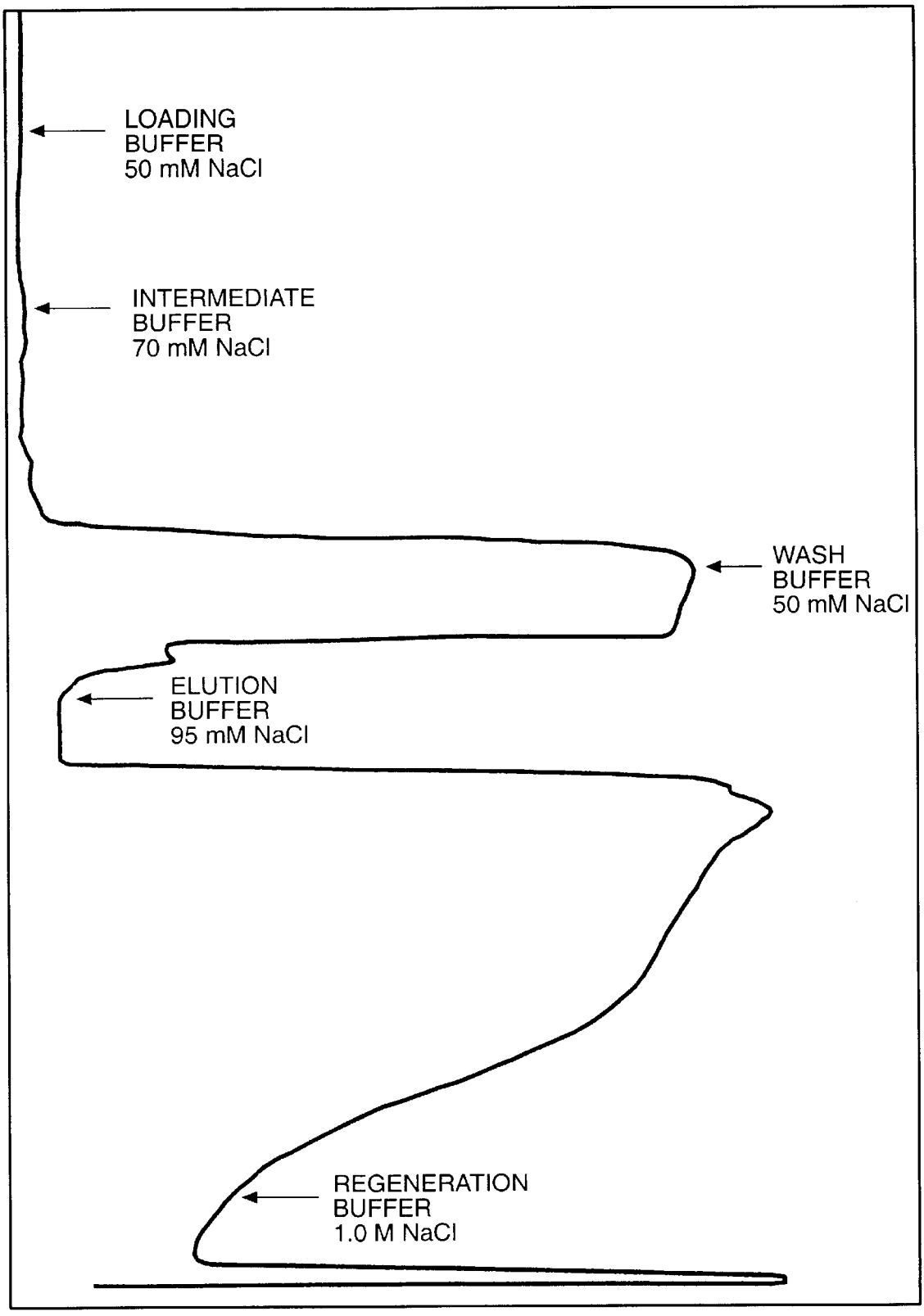
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
7.6 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
7.0 pH)
STEP E
REGENERATE
ION EXCHANGER
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._2


LOADING
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WASH
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ELUTION
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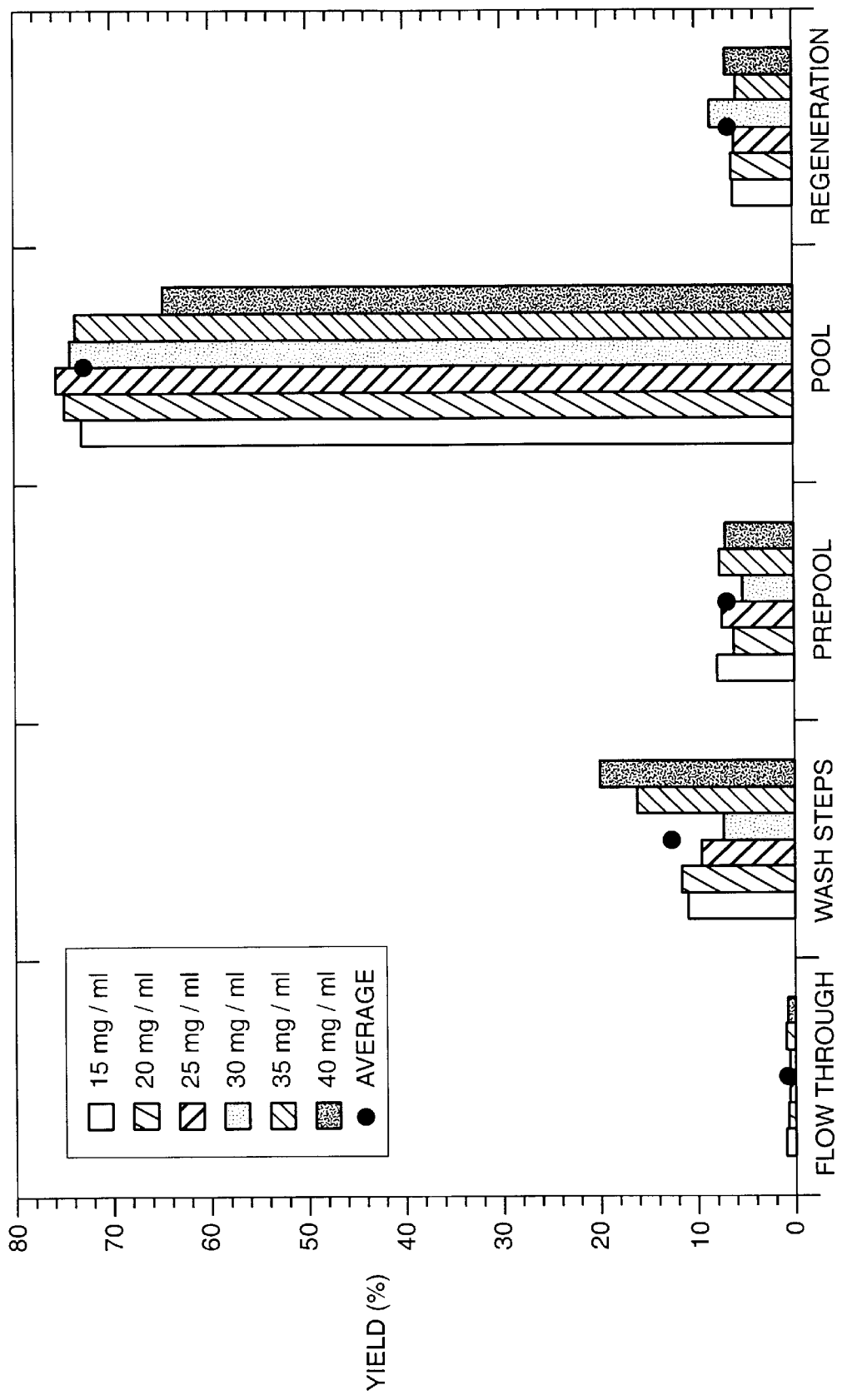
95 mM NaCl
REGENERATION
BUFFER
1.0 M NaCl

FIG._3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0
10
20
30
40
50
60
70
80
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
AVERAGE
FLOW THROUGH
WASH STEPS
PREPOOL
POOL
REGENERATION
CHROMATOGRAPHY FRACTION

FIG._4

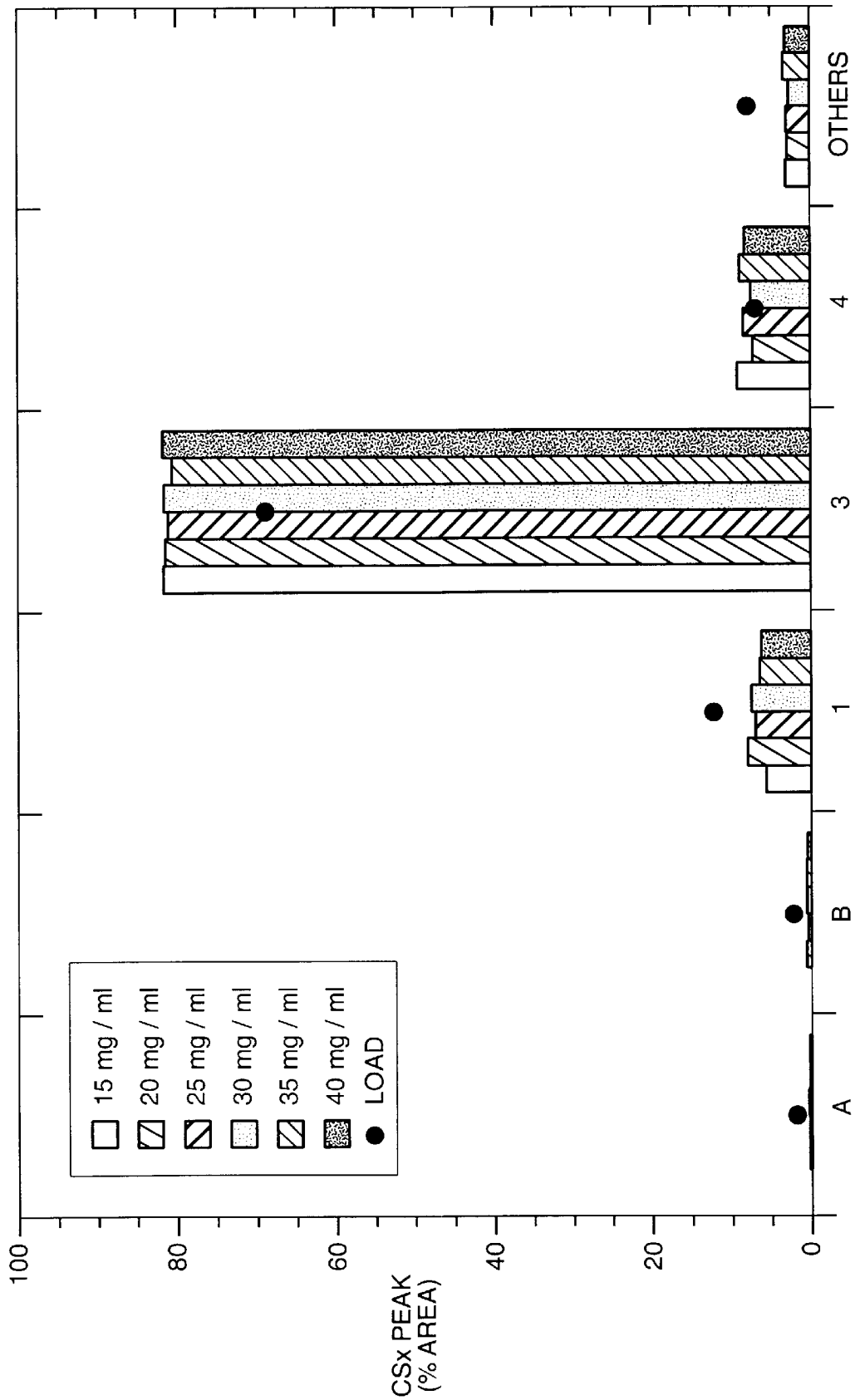
CSx PEAK (% AREA)
100
80
60
40
20
0
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
LOAD
A
B
1
3
4
OTHERS
CSx PEAK

FIG._5

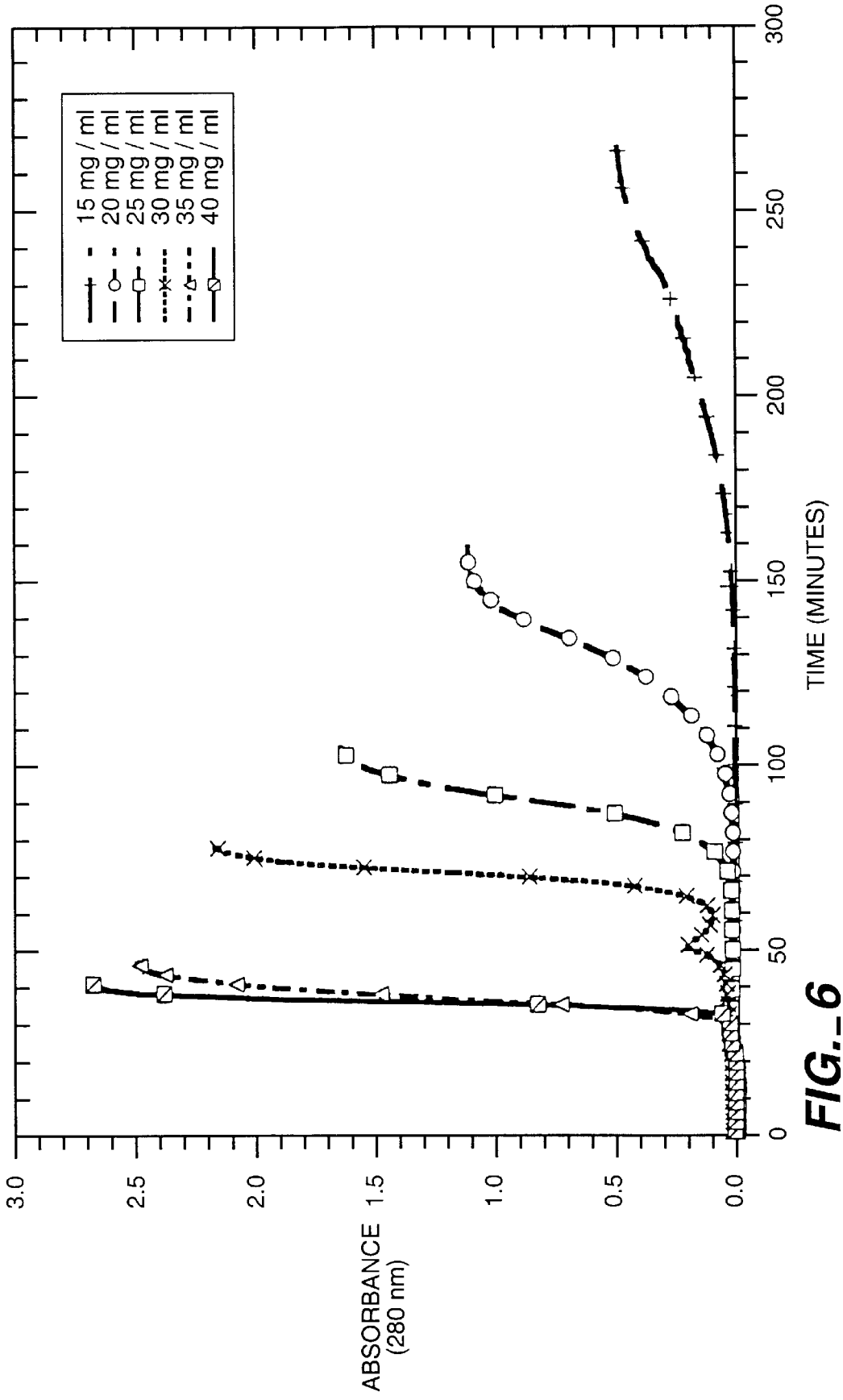
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
ABSORBANCE (280 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
TIME (MINUTES)

FIG._6

LIGHT CHAIN

```
1   D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q D V N T A V A W Y Q Q K P G K A P K
46  L L I Y S A S F L Y S G V P S R F S G S R S G T D F T L T I S S L Q P E D F A T Y Y C Q Q
91  H Y T T P P T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L
136 L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T
181 L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
```

FIG._7A

HEAVY CHAIN

1 (15, 30, 45)
E V Q L V E S G G G L V Q P G G S L R L S C A A S G F N I K D T Y I H W V R Q A P G K G L

46 (60, 75, 90)
E W V A R I Y P T N G Y T R Y A D S V K G R F T I S A D T S K N T A Y L Q M N S L R A E D

91 (105, 120, 135)
T A V Y Y C S R W G G D G F Y A M D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S

136 (150, 165, 180)
K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S

181 (195, 210, 225)
G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K

226 (240, 255, 270)
T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S

271 (285, 300, 315)
H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D

316 (330, 345, 360)
W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E

361 (375, 390, 405)
M T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G

406 (420, 435, 449)
S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G

FIG._7B

PROTEIN PURIFICATION

This is a, non-provisional application claiming priority under 35 USC §119 to provisional application No. 60/084,459 filed May 6, 1998, the entire disclosure of which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to protein purification. In particular, the invention relates to a method for purifying a polypeptide (e.g. an antibody) from a composition comprising the polypeptideand at least one contaminant using the method of ion exchange chromatography.

2. Description of Related Art

The large-scale, economic purification of proteins is increasingly an important problem for the biotechnology industry. Generally, proteins are produced by cell culture, using either mammalian or bacterial cell lines engineered to produce the protein of interest by insertion of a recombinant plasmid containing the gene for that protein. Since the cell lines used are living organisms, they must be fed with a complex growth medium, containing sugars, amino acids, and growth factors, usually supplied from preparations of animal serum. Separation of the desired protein from the mixture of compounds fed to the cells and from the by-products of the cells themselves to a purity sufficient for use as a human therapeutic poses a formidable challenge.

Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves: lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through".

Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins. In ion exchange, chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; i.e., the pH or conductivity is increased or decreased in a single direction.

SUMMARY OF THE INVENTION

The present invention provides an ion exchange chromatographic method wherein a polypeptide of interest is bound to the ion exchange material at an initial conductivity or pH and then the ion exchange material is washed with an intermediate buffer at a different conductivity or pH, or both. At a specific point following this intermediate wash, and contrary to ion exchange chromatography standard practice, the ion exchange material is washed with a wash buffer where the change in conductivity or pH, or both, from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity or pH, or both, achieved, in the previous steps. Only after washing with the wash buffer, is the ion exchange material prepared for the polypeptide molecule of interest to be eluted by the application of the elution buffer having a conductivity or pH, or both, which differ from the conductivity or pH, or both, of the buffers used in previous steps.

This novel approach to ion exchange chromatography is particularly useful in situations where a product molecule must be separated from a very closely related contaminant molecule at full manufacturing scale, where both purity and high recovery of polypeptide product are desired.

Accordingly, the invention provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to an ion exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the ion exchange material with an intermediate buffer at a second conductivity and/or pH so as to elute the contaminant from the ion exchange material;

(c) washing the ion exchange material with a wash buffer which is at a third conductivity and/or pH, wherein the change in conductivity and/or pH from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity and/or pH from the loading buffer to the intermediate buffer; and

(d) washing the ion exchange material with an elution buffer at a fourth conductivity and/or pH so as to elute the polypeptide from the ion exchange material. The first conductivity and/or pH may be the same as the third conductivity and/or pH.

Where the ion exchange material comprises a cation exchange resin, the conductivity and/or pH of the intermediate buffer is/are preferably greater than the conductivity and/or pH of the loading buffer; the conductivity and/or pH of the wash buffer is/are preferably less than the conductivity and/or pH of the intermediate buffer; and the conductivity and/or pH of the elution buffer is/are preferably greater than the conductivity and/or pH of the intermediate buffer. Preferably, the conductivity and/or pH of the wash buffer is/are about the same as the conductivity and/or pH of the loading buffer.

Preferably elution of the contaminant and of the polypeptide is achieved by modifying the conductivity of the intermediate buffer and of the elution buffer, respectively, while keeping the pH of these buffers approximately the same.

The invention also provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to a cation exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the cation exchange material with an intermediate buffer at a second conductivity and/or pH which is greater than that of the loading buffer so as to elute the contaminant from the ion exchange material;

(c) washing the cation exchange material with a wash buffer which is at a third conductivity and/or pH which is less than that of the intermediate buffer; and

(d) washing the cation exchange material with an elution buffer at a fourth conductivity and/or pH which is greater than that of the intermediate buffer so as to elute the polypeptide from the ion exchange material.

In addition, the invention provides a method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from about 20 mg to about 35 mg of the antibody per mL of cation exchange resin and, optionally, further comprising eluting the antibody from the cation exchange resin. The method preferably further comprises an intermediate wash step for eluting one or more contaminants from the ion exchange resin. This intermediate wash step usually precedes the step of eluting the antibody.

The invention further provides a composition comprising a mixture of anti-HER2 antibody and one or more acidic variants thereof, wherein the amount of the acidic variant(s) in the composition is less than about 25% and preferably less than about 20%, e.g. in the range from about 1% to about 18%. Optionally, the composition further comprises a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flow diagram showing how one could perform cation exchange chromatography by altering conductivity (e.g. to the NaCl concentrations of Example 1 below) or by altering pH (e.g. to the pH values as shown in the flow diagram).

FIG. **2** is a flow diagram showing how one could perform anion exchange; chromatography by altering conductivity (e.g. to the NaCl concentrations as depicted in the figure) or by altering pH (e.g. to the pH values as shown).

FIG. **3** is an absorbance trace from a cation exchange chromatography run of Example 1 at full manufacturing scale. Points at which the column is washed with the different buffers described herein are marked with arrows.

FIG. **4** depicts recombinant humanized anti-HER2 monoclonal antibody (rhuMAb HER2) recovered in each chromatography fraction (calculated as the percentage of the sum total of all fractions of the relevant chromatography). Flow through, wash steps, and prepool fractions: are all effluent samples collected from the onset of load to the initiation of pooling. The pool fraction is the five column volume effluent sample of elution starting at the leading shoulder's inflection point. The regeneration fraction contains effluent captured from the end of pooling to the end of regeneration.

FIG. **5** shows the quality of rhuMAb HER2 in each cation exchange chromatography pool sample as evaluated by carboxy sulfon cation exchange high pressure liquid chromatography (CSx HPIEX). Peaks a, b, and 1 are deamidated forms of rhuMAb HER2. Peak 3 is nondeamidated rhuMAb HER2. Peak 4 is a combination of C-terminal Lysine containing and iso-aspartate variants of rhuMAb HER2.

FIG. **6** shows the absorbance (280 nm) profiles of the 0.025 M MES/0.070 M NaCl, pH 5.6 wash for each chromatography. The mass of rhuMAb HER2 applied to the cation exchange resin effects the peak's absorbance level at the apex as well as the amount of buffer required to reach the apex. Due to minor peaks which occur (as best seen in the 30 mg/mL load) in this wash, the apex is defined as absorbance levels of at least 0.5 absorbance units (AU).

FIGS. **7**A and **7**B show the amino acid sequences of humMAb4D5-8 light chain (SEQ ID NO:1) and humMAb4D5-8 heavy chain (SEQ ID NO:2), respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The "composition" to be purified herein comprises the polypeptlide of interest and one or more contaminants. The composition may be "partially purified" (i.e. having been subjected to one or more purification steps, such as Protein A Chromatography as in Example 1 below) or may be obtained directly from a host cell or organism producing the polypeptide (e.g. the composition may comprise harvested cell culture fluid).

As used herein, "polypeptide" refers generally to peptides and proteins having more than about ten amino acids. Preferably, the polypeptide is a mammalian protein, examples of which include renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones: or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β**1**, TGF-β**2**, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins (IGFBPs); CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive fac-

tors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides. Most preferred is a full length antibody that binds human HER2.

A "contaminant" is a material that is different from the desired polypeptide product. The contaminant may be a variant of the desired polypeptide (e.g. a deamidated variant or an aminoaspartate variant of the desired polypeptide) or another polypeptide, nucleic acid, endotoxin etc.

A "variant" or "amino acid sequence variant" of a starting polypeptide is a polypeptide that comprises an amino acid sequence different from that of the starting polypeptide. Generally, a variant will possess at least 80% sequence identity, preferably at least 90% sequence identity, more preferably at least 95% sequence identity, and most preferably at least 98% sequence identity with the native polypeptide. Percentage sequence identity is determined, for example, by the Fitch et al., *Proc. Natl. Acad. Sci. USA* 80:1382–1386 (1983), version of the algorithm described by Needleman et al., *J. Mol. Biol.* 48:443–453 (1970), after aligning the sequences to provide for maximum homology. Amino acid sequence variants of a polypeptide may be prepared by introducing appropriate nucleotide changes into DNA encoding the polypeptide, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites. Methods for generating amino acid sequence variants of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

An "acidic variant" is a variant of a polypeptide of interest which is more acidic (e.g. as determined by cation exchange chromatography) than the polypeptide of interest. An example of an acidic variant is a deamidated variant.

A "deamidated" variant of a polypeptide molecule is a polypeptide wherein one or more asparagine residue(s) of the original polypeptide have been converted to aspartate, i.e. the neutral amide side chain has been converted to a residue with an overall acidic character. Deamidated humMAb4D5 antibody from the Example below has Asn30 in CDR1 of either or both of the V_L regions thereof converted to aspartate. The term "deamidated human DNase" as used herein means human DNase that is deamidated at the asparagine residue that occurs at position 74 in the amino acid sequence of native mature human DNase (U.S. Pat. No. 5,279,823; expressly incorporated herein by reference).

The term "mixture" as used herein in reference to a composition comprising an anti-HER2 antibody, means the presence of both the desired anti-HER2 antibody and one or more acidic variants thereof. The acidic variants may comprise predominantly deamidated anti-HER2 antibody, with minor amounts of other acidic variant(s). It has been found, for example, that in preparations of anti-HER2 antibody obtained from recombinant expression, as much as about 25% of the anti-HER2 antibody is deamidated.

In preferred embodiments of the invention, the polypeptide is a recombinant polypeptide. A "recombinant polypeptide" is one which has been produced in a host cell which has been transformed or transfected with nucleic acid encoding the polypeptide, or produces the polypeptide as a result of homologous recombination. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing nucleic acid into a cell. Following transformation or transfection, the nucleic acid may integrate into the host cell genome, or may exist as an extrachromosomal element. The "host cell" includes a cell in in vitro cell culture as well a cell within a host animal. Methods for recombinant production of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

The antibody herein is directed against an "antigen" of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include; those polypeptides discussed above. Preferred molecular targets for antibodies encompassed by the present invention include CD polypeptides such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and av/b3 integrin including either a or b subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; polypeptide C etc. Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The

modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). In a further embodiment, "monoclonal antibodies" can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552–554 (1990). Clackson et al., *Nature*, 352:624–628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779–783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265–2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255–258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24–34 (L1), 50–56 (L2) and 8997 (L3) in the light chain variable domain and 31–35 (H1), 50–65 (H2) and 95–102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Polypept ides of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. residues 26–32 (L1), 50–52 (L2) and 91–96 (L3) in the light chain variable domain and 26–32 (H1), 53–55 (H2) and 96–101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901–917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The CDR and FR residues of the rhuMAb HER2 antibody of the example below (humAb4D5-8) are identified in Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from nonhuman immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general the humanized antibody will comprise substantially all of at leastone, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglbbulin constant region (Fc), typically that of a human immunoglobulin.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products, using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis, of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab',

$F(ab')_2$, and Fv fragment's; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107–117 (1992) and Brennan et al., *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab**40** -SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163–167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185. "Single-chain Fv" or "sFv" antibody, fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269–315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H—V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90:6444–6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata et al. *Polypeptide Eng.* 8(10):1057–1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H—C_H1—V_H—C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

"Multispecific antibodies" have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-$p185^{HER2}$/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-$p185^{HER2}$, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell ahesion molecule (NCAM)/anti-CD3, anti-folate binding protein. (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor-antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD76/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. FcγRI, or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-$p185^{HER2}$/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, antiHRP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537–539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829; and in Traunecker et al., *EMBO J.*, 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the .desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present .in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy

chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217–225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab fragment was separately secretedfrom *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547–1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The diabody technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444–6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

The phrase "ion exchange material" refers to a solid phase which is negatively charged (i.e. a cation exchange resin) or positively charged (i.e. an anion exchange resin). The charge may be provided by attaching one or more charged ligands to the solid phase, e.g. by covalent linking. Alternatively, or in addition, the charge may be an inherent property of the solid phase (e.g. as is the case-for silica, which has an overall negative charge).

By "solid phase" is meant a non-aqueous matrix to which one or more charged ligands can adhere. The solid phase may be a purification column, a discontinuous phase of discrete particles, a membrane, or filter etc. Examples of materials for forming the solid phase include polysaccharides (such as agarose and cellulose); and other mechanically stable matrices such as silica (e.g. controlled pore glass), poly(styrenedivinyl)benzene, polyacrylamide, ceramic particles and derivatives of any of the above.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-ihethyl-cellulose, BAKERBOND ABX™, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from Pharmacia) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from Pharmacia).

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (Pharmacila).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in Buffers. *A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., Ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 5 to about 7 (e.g. as in Example 1 below). Examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more contaminants onto the ion exchange resin. The loading buffer .has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more contaminants) is/are bound to the ion exchange resin.

The "intermediate buffer" is used to elute one or more contaminants from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that the contaminant is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elutibn buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all contaminants and the polypeptide of interest from the ion exchange resin.

The term "conductivity" refers to the ability of an aqueous solution to conduct an electric current between two electrodes. In solution, the current flows by ion transport. Therefore, with an increasing amount of ions present in the aqueous solution, the solution will have a higher conductivity. The unit of measurement for conductivity is mmhos (mS/cm), and can be measured using a conductivity meter sold, e.g., by Orion. The conductivity of a solution may be altered by changing the concentration of ions therein. For example, the concentration of a buffering agent and/or concentration of a salt (e.g. NaCl or KCl) in the solution may be altered in order to achieve the desired conductivity. Preferably, the salt concentration of the various buffers is modified to achieve the desired conductivity as in the Example below.

By "purifying" a polypeptide from a composition comprising the polypeptide and one or more contaminants is meant increasing the degree of purity of the polypeptide in the composition by removing (completely or partially) at least one contaminant from the composition. A "purification step" may be part of an overall purification process resulting in a "homogeneous" composition, which is used herein to refer to a composition comprising at least about 70% by weight of the polypeptide of interest, based on total weight of the composition, preferably at least about 80% by weight.

Unless indicated otherwise, the term "HER2" when used herein refers to human HER2 protein and "HER2" refers to human HER2 gene. The human HER2 gene and HER2 protein are described in Semba et al., *PNAS* (*USA*) 82:6497–6501 (1985) and Yamamoto et al. *Nature* 319:230–234 (1986) (Genebank accession number X03363), for example.

The term "humMAb4D5-8" when used herein refers to a humanized anti-HER2 antibody comprising the light chain amino acid sequence of SEQ ID NO:1 and the heavy chain amino acid sequence of SEQ ID NO:2 or amino acid sequence variants thereof which retain the ability to bind HER2 and inhibit growth of tumor cells which overexpress HER2 (see U.S. Pat. No. 5,677,171; expressly incorporated herein by reference).

The "pI" or "isoelectric point" of a polypeptide refer to the pH at which the polypeptide's positive charge balances its negative charge pI can be calculated from the net charge of the amino acid residues of the polypeptide or can be determined by isoelectric focussing (e.g. using CSx chromatography as in the Example below).

By "binding" a molecule to an ion exchange material is meant exposing the molecule to the ion exchange material under appropriate conditions (pH/conductivity) such that the molecule is reversibly immobilized in or on the ion exchange material by virtue of ionic interactions between the molecule and a charged group or charged groups of the ion exchange material.

By "washing" the ion exchange material is meant passing an appropriate buffer through or over the ion exchange material.

To "elute"l a molecule (e.g. polypeptide or contaminant) from an ion exchange material is meant to remove the molecule therefrom by altering the ionic strength of the buffer surrounding the ion exchange material such that the buffer competes with the molecule for the charged sites on the ion exchange material.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as-well as those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with the polypeptide purified as described herein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

The word "label" when used herein refers to a detectable compounder composition which is conjugated directly or indirectly to the polypeptide. The label may be itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL , Bristol-Myers Squibb Oncology, Princeton, N.J.), and doxetaxel, toxotere, methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

Modes for Carrying Out the Invention

The invention herein provides a method for purifying a polypeptide from a composition (e.g. an aqueous solution) comprising the polypeptide and one or more contaminants. The composition is generally one resulting from the recom-

binant production of the polypeptide, but may be that resulting from production of the polypeptide by peptide synthesis (or other synthetic means) or the polypeptide may be purified from a native source of the polypeptide. Preferably the polypeptide is an antibody, e.g. one which binds the HER2 antigen.

For recombinant production of the polypeptide, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide is readily isolated and sequenced using conventional procedures (e.g., where the polypeptide is an antibody by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (e.g. as described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., *E. coli*, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., *Salmonella typhimurium*, Serratia, e.g., *Serratia marcescans*, and Shigella, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as *P. aeruginosa*, and Streptomyces. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; Kluyveromyces hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltil* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; yarrowia (EP 402,226); *Pichia pastoris* (EP 183,070); Candida; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; Schwanniomyces such as Schwanniomyces occidentalis; and filamentous fungi such as, e.g., Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese ihamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243–251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44–68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM); (Sigma), RPMI-1640 (Sigma), and Dulbeccols Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), suffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (e.g. resulting from homogenization), is removed, for example, by centrifugation or ultrafiltration. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

The polypeptide is then subjected to one or more purification steps, including the ion exchange chromatography

method as claimed herein. Examples of additional purification procedures which may be performed prior to, during, or following the ion exchange chromatography method include fractionation on a hydrophobic interaction chromatography (e.g. on phenyl sepharose), ethanol precipitation, isoelectric focusing, Reverse Phase HPLC, chromatography on silica, chromatography on HEPARIN SEPHAROSET, further anion exchange chromatography and/or further cation exchange chromatography, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography (e.g. using protein A, protein G, an antibody, a specific substrate, ligand or antigen as the capture reagent).

Ion exchange chromatography is performed as claimed herein. A decision is first made as to whether an anion or cation exchange resin is to be employed. In general, a cation exchange resin may be used for polypeptides with pIs greater than about 7 and an anion exchange resin may be used for polypeptides with pIs less than about 7.

The anion or cation exchange resin is prepared according to known methods. Usually, an equilibration buffer is passed through the ion exchange resin prior to loading the composition comprising the polypeptide and one or more contaminants onto the resin. Conveniently, the equilibration buffer is the same as the loading buffer, but this is not required.

The various buffers used for the chromatography depend, for example, on whether a cation or anion exchange resin is employed. This is shown more-clearly in the flow diagrams of FIGS. **1** and **2**.

With particular reference to FIG. **1**, which shows exemplary steps to be performed where a cation exchange resin is used, the pH and/or conductivity of each buffer is/are increased relative to the preceding buffer, except for the wash buffer where the conductivity and/or pH is/are less than the conductivity and/or pH of the preceding intermediate buffer. The aqueous solution comprising the polypeptide of interest and contaminant(s) is loaded onto the cation exchange resin using the loading buffer that is at a pH and/or conductivity such that the polypeptide and the contaminant bind to the cation exchange resin. As in the Example below, the loading buffer may be at a first low conductivity (e.g. from about 5.2 to about 6.6 mmhos). An exemplary pH for the loading buffer may be about 5.0 (see FIG. **1**). From about 20 mg/mL to about 35 mg/mL of the polypeptide (e.g. of a full length antibody) may, for example, be loaded on the ion exchange resin.

The cation exchange resin is then washed with an intermediate buffer which is at a second conductivity and/or pH so as to essentially elute the contaminant, but not a substantial amount of the polypeptide of interest. This may be achieved by increasing the conductivity or pH, or both, of the intermediate buffer. The change from loading buffer to intermediate buffer may be step-wise or gradual as desired. In the Example herein, the intermediate buffer had a greater conductivity than that of the loading buffer (i.e. the intermediate buffer's conductivity was in the range from about 7.3 to about 8.4 mmhos). Alternatively, as shown in FIG. **1**, the pH of the intermediate buffer may exceed that of the loading buffer in this embodiment of the invention, where a cation exchange resin is used. For example, the intermediate buffer may have a pH of about 5.4.

Following washing with the intermediate buffer, the cation exchange resin is washed or re-equilibrated with the wash buffer which has a conductivity or pH, or both, which is/are less than that of the intermediate buffer (i.e. the conductivity, or pH, or both, is/are changed in an opposite, i.e. reverse, direction to the preceding step, unlike ion exchange chromatography steps in the literature). In the Example below, the wash buffer had about the same conductivity as the loading buffer (i.e. in the range from about 5.2 to about 6.6 mmhos) and its conductivity was, therefore, less than that of the intermediate buffer. In another embodiment, one may reduce the conductivity of the wash buffer to a conductivity that is less than, or greater than, that of the loading buffer, provided the conductivity of the wash buffer is less than that of the intermediate buffer. In another embodiment, the pH of the wash buffer may be less than the pH of the intermediate buffer (e.g. the pH of the wash buffer may about 5.0). The change in conductivity and/or pH of the wash buffer compared to the intermediate buffer may be achieved by step-wise or gradual change of either or both of these parameters.

After the wash step of the preceding paragraph, the cation exchange resin is prepared for elution of the desired polypeptide molecule therefrom. This is achieved using an elution buffer that has a pH and/or conductivity such that the desired polypeptide no longer binds to the cation exchange resin and therefore is eluted therefrom. The pH and/or conductivity of the elution buffer generally exceed(s) the pH and/or conductivity of the loading buffer, the intermediate buffer and the wash buffer used in the previous steps. In the Example below, the conductivity of the elution buffer was in the range from about 10.0 to about 11.0 mmhos. Alternatively, or in addition, the pH of the elution buffer may be increased relative to the wash buffer and to the intermediate buffer (for example, the pH of the elution buffer may about 6.0). The change in conductivity and/or pH may be step-wise or gradual, as desired. Hence, the desired polypeptide is retrieved from the cation exchange resin at this stage in the method.

In an alternative embodiment, the ion exchange material comprises an anion exchange resin. This embodiment of the invention is depicted in FIG. **2** herein. As illustrated in this figure, the changes in conductivity are generally as described above with respect, to a cation exchange resin. However, the direction of change fin pH is different for an anion exchange resin. For example, if elution of the contaminant(s) and polypeptide are to be achieved by altering pH, the loading buffer has a first pH and the pH is decreased in the intermediate buffer so as to elute the contaminant or contaminants. In the third step, the column is washed/re-equilibrated with the wash buffer and the change in conductivity or pH, or both, is in the opposite direction to that of the previous step. Hence, the pH may be increased in the wash buffer, compared to the intermediate buffer. Following this step, the polypeptide of interest is eluted from the anion exchange resin using an elution buffer at a fourth conductivity and/or pH. If pH is altered, it will normally be less than the pH of the loading buffer, the intermediate buffer and the wash buffer. The change in pH and/or conductivity in progressive buffers can, as explained above, be step-wise or gradual.

In the preferred embodiment of the invention, a single parameter (i.e. either conductivity or pH) is changed to achieve elution of both the polypeptide and contaminant, while the other parameter (i.e. pH or conductivity, respectively) remains about constant. For example, while the conductivity of the various buffers (loading buffer, intermediate buffer, wash buffer and/or elution buffer) may differ, the pH's thereof may be essentially the same.

In, an optional embodiment of the invention, the ion exchange resin is regenerated with a regeneration buffer

after elution of the polypeptide, such that the column can be re-used. Generally, the conductivity and/or pH of the regeneration buffer is/are such that substantially all contaminants and the polypeptide of interest are eluted from the ion exchange resin. Generally, the regeneration buffer has a very high conductivity for eluting contaminants and polypeptide from the ion exchange resin.

The method herein is particularly useful for resolving a polypeptide molecule of interest from at least one contaminant, where the contaminant and polypeptide molecule of interest differ only slightly in ionic charge. For example, the pIs of the polypeptide and contaminant may be only "slightly different", for example they may differ by only about 0.05 to about 0.2 pI units. In the Example below, this method could be used to resolve an anti-HER2 antibody having a pI of 8.87, from a singly-deamidated variant thereof having a pI of 8.79. Alternatively, the method may be used to resolve a deamidated DNase, for example, from nondeamidated DNase. In another embodiment, the method may be used to resolve a polypeptide from a glycosylation variant thereof, e.g. for resolving a variant of a polypeptide having a different distribution of sialic acid compared to the nonvariant polypeptide.

The polypeptide preparation obtained according to the ion exchange chromatography method herein may be subjected to additional purification steps, if necessary. Exemplary further purification steps have been discussed above.

Optionally, the polypeptide is conjugated to one or more heterologous molecules as desired. The heterologous molecule may, for example, be one which increases the serum half-life of the polypeptide (e.g. polyethylene glycol, PEG), or it may be a label (e.g. an enzyme, fluorescent label and/or radionuclide) or a cytotoxic molecule (e.g. a toxin, chemotherapeutic drug, or radioactive isotope etc).

A therapeutic formulation comprising the polypeptide, optionally conjugated with a heterologous molecule, may be prepared by mixing the polypeptide having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. "Pharmaceutically acceptable" carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins:, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). The humMAb4D5-8 antibody of particular interest herein may be prepared as a lyophilized formulation, e.g. as described in WO 97/04801; expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. For example, for an anti-HER2 antibody a chemotherapeutic agent, such as a taxoid or tamoxifen, may be added to the formulation.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulation to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide variant, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly (vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and y ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and go leuprolide acetate), and poly-D-(–)-3-hydroxybutyric acid.

The polypeptide purified as disclosed herein or the composition comprising the polypeptide and a pharmaceutically acceptable carrier is then used for various diagnostic, therapeutic or other uses known for such polypeptides and compositions. For example, the polypeptide may be used to treat a disorder in a mammal by administering a therapeutically effective amount of the polypeptide to the mammal.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1

Full length human IgG rhuMAb HER2 (humAb4D5-8 in Carter et al. *Proc. Natl. Acad. Sci.* 89: 4285–4289 (1992) comprising the light chain amino acid sequence of SEQ ID NO:1 and heavy chain amino acid sequence of SEQ ID NO:2) was produced recombinantly in CHO cells. Following protein production and secretion to the cell culture medium, the CHO cells were separated from the cell culture medium by tangential flow filtration (PROSTACK™). Protein A chromatography was then performed by applying the Harvested Cell Culture Fluid (HCCF) from the CHO cells directly to an equilibrated PROSEP A™ column (Bioprocessing, Ltd).

Following Protein A chromatography, cation exchange chromatography was performed using a sulphopropyl (SP)-SEPHAROSE FAST FLOW™ (SPSFF) column (Pharmacia) to further separate the desiredanti-HER2 antibody molecule. The chromatography operation was performed in bind and elute mode.

The SPSFF column was prepared for load by sequential washes with regeneration buffer (0.025 M MES/1.0 M NaCl,

pH 5.6) followed by equilibration buffer (0.025 M MES/50 mM NaCl, pH 5.6). The column was then loaded with Protein A pool adjusted to a pH of 5.60±0.05 and a conductivity of 5.8±0.2 mmhos. Prior to elution, the column was washed in three steps: (1) loading buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1 column volume; (2) intermediate buffer (0.025 M MES/70 mM NaCl, pH 5.6) until an apex of a 280 nm peak was reached; and (3) wash buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1.2 column volumes rhuMAb HER2 was then eluted from the column with elution buffer (0.025 M MES/95 mM NaCl, pH 5.6). The elution 280 nm profile has a shoulder on the leading edge (FIG. **3**). At the inflection point of this shoulder, pooling starts and continues for an additional 5 column volumes. The column was then regenerated with regeneration buffer (0.025 M MES/1.0 M NaCl, pH 5.6).

Materials and Methods

Column and Load Preparation: A reduced-scale SPSFF column was packed. The dimensions were: 27.0 mL volume, 1.0 cm diameter and 34.5 cm bed height. The pH of an aliquot of Protein A pool was titered to 5.6 with 1.5 M Tris base. The conductivity of the pool was reduced by the addition of an equal volume of sterile water for injection (SWFI).

Chromatography: The chromatography runs for this study were performed with Pharmacia's UNICORN™ FPLC system. The equilibration, load, and initial wash steps were performed at a linear flow rate of 200 cm/h. All chromatography steps were performed at a linear flow rate of 100 cm/h. The sequence of chromatography steps are defined in Table 1. A total of six chromatography runs were performed with load densities of 15, 20, 25, 30, 35, and 40 mg of rhuMAb HER2 per mL of SPSFF resin.

TABLE 1

Chromatography Steps[1]

Chromatography Step	Buffer	Endpoint
Equilibration: Part 1	0.025 M MES/1.0 M NaCl, pH 5.6	2 CV[2]
Equilibration: Part 2	0.025 M MES/0.05 M NaCl, pH 5.6	pH: 5.6 ± 0.1 Cond.: 5.8 ± 0.2 mmhos
Load	Adjusted Protein A Pool	As Required
Wash 1	0.025 M MES/0.05 M NaCl, pH 5.6	1.5 CV
Wash 2	0.025 M MES/0.07 M NaCl, pH 5.6	Apex of Peak
Wash 3	0.025 M MES/0.05 M NaCl, pH 5.6	2 CV
Elution: Prepool	0.025 M MES/0.095 M NaCl, pH 5.6	To Leading Shoulder's Inflection Point (~1.2 CV)
Elution: Pool	0.025 M MES/0.095 M NaCl, pH 5.6	5 CV
Regeneration	0.025 M MES/1.0 M NaCl, pH 5.6	2 CV

[1]The equilibration of the resin was performed in manual mode; the remaining steps were executed from a Pharmacia Unicorn Program.
[2]CV = column volume(s).

Total Protein: The protein concentration of each chromatography fraction (flow through, wash steps, elution prepool, elution pool, and regeneration) was determined by spectrophometric scans of each sample. The results were used to calculate product recovery yields. The extinction coefficient for rhuMAb HER2 is 1.45. Calculations used to derive the results (FIG. **4**) are:

$$\text{Protein Concentration (mg/mL)} = \frac{280\text{ nm}}{1.45} \times \text{Dilution Factor}$$

$$\text{Protein Mass (mg) in Each Fraction} = \text{Protein Concentration (mg/mL)} \times \text{Fraction Volume (mL)}$$

$$\text{Yield (\%)} = \frac{\text{Fraction Mass (mg)}}{\text{Total Mass (mg)}} \times 100$$

Determination of rhuMAb HER2 Antibody Variants (CSx HPIEX): The rhuMAb HER2 SPSFF chromatography column resolves antibody variants. Fractions from each of the study chromatographies were tested for the relative amount of variant antibody by CSx HPIEX chromatography. A BAKERBOND WIDE-PORETM CSx HPIEX column (4.6× 250 mm) was run at 1 mL/min at 55° C. The mobile phase was formed from a tertiary gradient (Table 2).

TABLE 2

Gradient Scheme

Time (min)	% A	% B	% C
0 - Initial Conditions	49	1	50
10.0	40	10	50
50.0	33	17	50
50.2	49	1	50
70.0	49	1	50

The column is run at 1 mL/min at 55° C.

The A buffer was 0.025 M MES, pH 5.9; the B buffer was 1 M Ammonium Acetate, pH 7.0; and the C solution was sterile water for injection. The column was equilibrated with the gradient's initial conditions (49% A; 1% B; and 50% C) and 200 μl of sample, diluted with SWFI and containing<300 μg protein, was injected. Each resulting chromatogram was integrated to determine the percent area of each peak for each fraction (Table 3 and FIG. **5**).

TABLE 3

CSx HPIEX analysis of rhuMAb HER2

CSx Peak	rhuMAb HER2 Variant
a & b	Light Chain: Asn → Asp[30] deamidation - and - Other unidentifiable variation by typtic map
1	Light Chain: Asn → Asp+ deamidation
3	Fully Processed Antibody
4	Heavy Chain: Asp → Iso-Asp[102] - and/or - Heavy Chain: An Additional Lys[450]
Others	Heavy Chain: Asp → Succinimide - and/or - Multiple permutations found in Peaks 1 and 4

Chromatograms Compared: The absorbance data (AU 280 nm) from each chromatography file was exported from Unicorn in ASCII format. The data from the 0.025 M MES/0.07 M NaCl, pH 5.6 wash was translated into Excel format and copied into KALEIDAGRAPH™. Using KALEIDAGRAPH™, the wash profiles were overlaid (FIG. **6**) and compared to each other.

RESULTS AND DISCUSSION

Deamidated and other acidic variants of rhuMAb HER2 were produced when the antibody was made by recombinant

DNA technology (see e.g., CSx peaks a, b and **1** in FIG. **5**). The deamidated and other acidic variants constituted about 25% (calculated as area under the integrated curve or profile obtained by CSx chromatography) of the composition obtained from the initial Protein A chromatography step. It was discovered that the ion exchange method described herein could be used to substantially reduce the amount of deamidated and other acidic variants in the anti-HER2 composition, i.e. to about 13% or less (i.e. the amount of acidic variants in the preparation subjected to cation exchange chromatography as described herein was decreased by about 50% or more).

An absorbance trace from a cation exchange column run performed as described above is shown in FIG. **3**. This method resolved a deamidated variant of anti-HER2 antibody that differed only slightly from nondeamidated anti-HER2 antibody. The increase in conductivity from the initial conditions to the intermediate wash began to elute the deamidated anti-HER2 antibody. However, continued washing at this conductivity was found to elute nondeamidated anti-HER2 antibody, resulting in a loss of product. Proceeding directly from the intermediate buffer to the elution buffer was observed to result in either an unacceptably low removal of deamidated anti-HER2 antibody from the product if pooling began early or unacceptably low yields of anti-HER2 antibody product if pooling was delayed until the deamidated anti-HER2 antibody was reduced. It was discovered that by going back to lower conductivity as used initially, the elution of deamidated anti-HER2 antibody continued, without significant anti-HER2 antibody product elution.

The effect of rhuMAb HER2 load on (a) buffer requirements, (b) product recovery in the pool, and (c) product quality in the pool was evaluated.

At load-densities of 15 mg/mL up to 35 mg/mL, the product yield in the elution pool is approximately 75%. For the load density of 40 mg/mL, the product yield in the pool dropped to 65% (FIG. **4**). This reduced recovery in the pool is largely attributed to an increased antibody in the two wash steps (at 70 mM NaCl and 50 mM NaCl, respectively).

The quality of rhuMAb HER2 in all the elution pools is equivalent as determined by CSx HPIEX analysis (FIG. **5**). Compared to the load material; there is an enrichment of the nondeamidated antibody: (Peak 3), no change in the amount Iso-Asp^{102} or Lys^{450} antibody (Peak 4), and a reduction of the amount of Asp^{30} deamidated antibody (Peaks a, b, 1 and others).

The quality of rhuMAb HER2 in these cation pools is improved through the intermediate wash step. As the mass of rhuMAb HER2 bound to the resin increases, the intermediate buffer volume consumption needed to reach the apex of the 280 nm peak decreases. The buffer volume required for a 40 mg/mL load density is approximately 2.5 column volumes. The buffer volume required for a 15 mg/mL load density is approximately 15 column volumes. The exact increase of buffer requirement is not linear with the 5 mg/mL incremental changes between these two extremes. The greatest increase is seen between, the load densities of 20 mg/mL and 15 mg/mL. Here the requirement doubles from 7.5 column volumes to the previously mentioned 15 column volumes of buffer. If the apex of the 70 mM NaCl wash peak is reached, however, the product quality is equivalent for any of load densities examined.

This study determined how much rhuMAb HER2 can be loaded onto the SPSFF resin. Between the ranges of 15 to 40 mg of antibody per mL of resin, there is no difference in the quality of rhuMAb HER2 recovered in the elution pool. The quantity of rhuMAb HER2 recovered, however, is reduced by approximately 10% when the resin is loaded with greater than 35 mg/mL. For consistent yields it is recommended that 35 mg/mL be set as the maximum load for manufacture of rhuMAb HER2. Furthermore, due to the substantial increase in the 70 mM NaCl wash volume requirement between the 20 and 15 mg/mL; it is recommended that 20 mg/mL be set as the minimal load for manufacture of rhuMAb HER2.

SEQUENCE LISTING

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<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
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-continued

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                80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
               110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
               125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
               140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
               155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
               170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
               185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
               200                 205                 210

Arg Gly Glu Cys


<210> SEQ ID NO 2
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<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
               20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
               35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
               50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
               65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
               80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
               95                 100                 105

Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
              110                 115                 120

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser
              125                 130                 135

Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys
              140                 145                 150

Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
              155                 160                 165

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
              170                 175                 180

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
              185                 190                 195

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
              200                 205                 210
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-continued

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Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
                215                 220                 225

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                230                 235                 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
                260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
                275                 280                 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
                290                 295                 300

Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
                305                 310                 315

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
                320                 325                 330

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
                335                 340                 345

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
                350                 355                 360

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
                365                 370                 375

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
                380                 385                 390

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
                395                 400                 405

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
                410                 415                 420

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
                425                 430                 435

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                440                 445
```

What is claimed is:

1. A method for purifying a polypeptide from a composition comprising the polypeptide and a deamidated variant thereof, which method comprises the following steps performed sequentially:

(a) binding the polypeptide and deamidated variant to an ion exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the ion exchange material with an intermediate buffer at a second conductivity and/or pH so as to elute the deamidated variant from the ion exchange material;

(c) washing the ion exchange material with a wash buffer which is at a third conductivity and/or pH, wherein the change in conductivity and/or pH from the intermediate buffer to the wash buffer is in an opposite

direction to the change in conductivity and/or pH from the loading buffer to the intersediate buffer; and

(d) washing the ion exchange material with an elution buffer at a fourth conductivity and/or pH so as to elute the polypeptide from the ion exchange material.

2. The method of claim **1** wherein the ion exchange material comprises a cation exchange resin.

3. The method of claim **2** wherein the conductivity and/or pH of the intermediate buffer is/are greater than the conductivity and/or pH of the loading buffer and the conductivity and/or pH of the wash buffer is/are less than the conductivity and/or pH of the intermediate buffer.

4. The method of claim **2** wherein the conductivity and/or pH of the elution, buffer is/are greater than the conductivity and/or pH of the intermediate buffer.

5. The method of claim **2** wherein the cation exchange resin comprises sulphopropyl immobilized on agarose.

6. The method of claim **1** wherein the ion exchange material comprises an anion exchange resin.

7. The method of claim **1** wherein the conductivity and/or pH of the wash buffer is/are about the same as the conductivity and/or pH of the loading buffer.

8. The method of claim **1** wherein elution of the deamidated variant and of the polypeptide is achieved by modifying the conductivity of the intermediate buffer and of the elution buffer, respectively.

9. The method of claim **8** herein the pH remains approximately constant for each of steps (a)–(d).

10. The method of claim **8** wherein the conductivity of the intermediate buffer and of the elution buffer is modified by changing the salt concentration therein.

11. The method of claim 10 wherein the conductivity of the intermediate buffer and of the elution buffer is modified by changing the NaCl concentration therein.

12. The method of claim 1 wherein the polypeptide and the deamidated variant have slightly different pIs.

13. The method of claim 1 further comprising washing the ion exchange material with a regeneration buffer after step (d).

14. The method of claim 1 wherein the polypeptide is an antibody.

15. The method of claim 14 wherein the antibody binds HER2.

16. The method of claim 15 wherein the antibody is humMAb4D5-8.

17. The method of claim 1 further comprising subjecting the composition comprising the polypeptide to one or more further purification steps either before, during, or after the ion exchange chromatography method so as to obtain a homogeneous preparation of the polypeptide.

18. The method of claim 17 further comprising conjugating the polypeptide with a heterologous molecule.

19. The method of claim 18 wherein the heterologous molecule is polyethylene glycol, a label or a cytotoxic agent.

20. The method of claim 17 further comprising preparing a pharmaceutical composition by combining the homogeneous preparation of the polypeptide with a pharmaceutically acceptable carrier.

21. A method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the antibody and contaminant to a cation exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the cation exchange material with an intermiediate buffer at a second conductivity and/or pH which is greater than that of the loading buffer so as to elute the contaminant from the cation exchange material;

(c) washing the cation exchange material with a wash buffer which is at a third conductivity and/or pH which is less than that of the intermediate buffer; and

(d) washing the cation exchange material with an elution buffer at a fourth conductivity and/or pH which is greater than that of the intermediate buffer so as to elute the antibody from the cation exchange material.

22. A method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the antibody and contaminant to an ion exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the ion exchange material with an intermediate buffer at a second conductivity and/or pH so as to elect the contaminant from the ion exchange material;

(c) washing the ion exchange material with a wash buffer which is at a third conductivity and/or pH, wherein the change in conductivity and/or pH from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity and/or pH from the loading buffer to the intermediate buffer; and

(d) washing the ion exchange material with an elution buffer at a fourth conductivity and/or pH so as to elute the antibody from the ion exchange material.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,489,447 B1
DATED : December 3, 2002
INVENTOR(S) : Basey et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 30,
Line 23, please delete “elect” and insert therefor -- elute --.

Signed and Sealed this

Twenty-second Day of April, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

EXHIBIT K

US009249218B2

(12) **United States Patent**
Basey et al.

(10) **Patent No.: US 9,249,218 B2**
(45) **Date of Patent: *Feb. 2, 2016**

(54) **PROTEIN PURIFICATION**

(75) Inventors: **Carol D. Basey**, Winters, CA (US); **Greg S. Blank**, Menlo Park, CA (US)

(73) Assignee: **GENENTECH, INC.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 214 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/313,931**

(22) Filed: **Dec. 7, 2011**

(65) **Prior Publication Data**

US 2014/0018523 A1 Jan. 16, 2014

Related U.S. Application Data

(60) Continuation of application No. 12/418,905, filed on Apr. 6, 2009, now abandoned, which is a continuation of application No. 11/398,447, filed on Apr. 5, 2006, now Pat. No. 7,531,645, which is a continuation of application No. 10/949,683, filed on Sep. 24, 2004, now Pat. No. 7,074,404, which is a continuation of application No. 10/253,366, filed on Sep. 24, 2002, now abandoned, which is a division of application No. 09/304,465, filed on May 3, 1999, now Pat. No. 6,489,447.

(60) Provisional application No. 60/084,459, filed on May 6, 1998.

(51) **Int. Cl.**

A61K 39/395	(2006.01)
A61K 39/00	(2006.01)
A61K 39/40	(2006.01)
C07K 16/28	(2006.01)
C07K 1/18	(2006.01)
C07K 16/06	(2006.01)
C07K 16/32	(2006.01)
A61K 38/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C07K 16/2863*** (2013.01); ***C07K 1/18*** (2013.01); ***C07K 16/065*** (2013.01); ***C07K 16/32*** (2013.01); *A61K 38/00* (2013.01); *C07K 2317/24* (2013.01); *C07K 2317/56* (2013.01)

(58) **Field of Classification Search**

USPC 424/130.1, 133.1, 138.1, 141.1, 152.1

See application file for complete search history.

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(57) **ABSTRACT**

A method for purifying a polypeptide by ion exchange chromatography is described which involves changing the conductivity and/or pH of buffers in order to resolve a polypeptide of interest from one or more contaminants.

7 Claims, 7 Drawing Sheets

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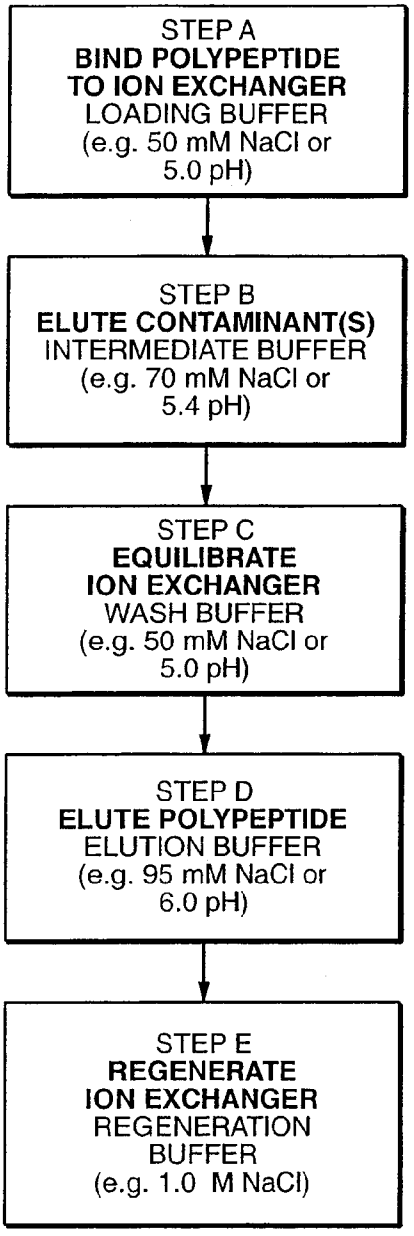
STEP A
BIND POLYPEPTIDE
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
5.4 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
5.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
6.0 pH)
STEP E
REGENERATE
ION EXCHANGER
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._1

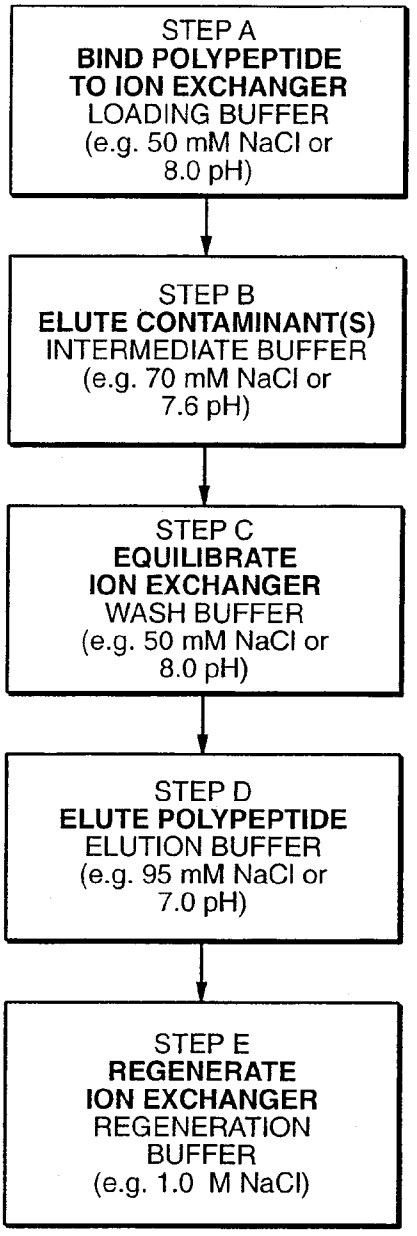
STEP A
BIND POLYPEPTIDE
TO ION EXCHANGER
LOADING BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP B
ELUTE CONTAMINANT(S)
INTERMEDIATE BUFFER
(e.g. 70 mM NaCl or
7.6 pH)
STEP C
EQUILIBRATE
ION EXCHANGER
WASH BUFFER
(e.g. 50 mM NaCl or
8.0 pH)
STEP D
ELUTE POLYPEPTIDE
ELUTION BUFFER
(e.g. 95 mM NaCl or
7.0 pH)
STEP E
REGENERATE
ION EXCHANGER
REGENERATION
BUFFER
(e.g. 1.0 M NaCl)

FIG._2

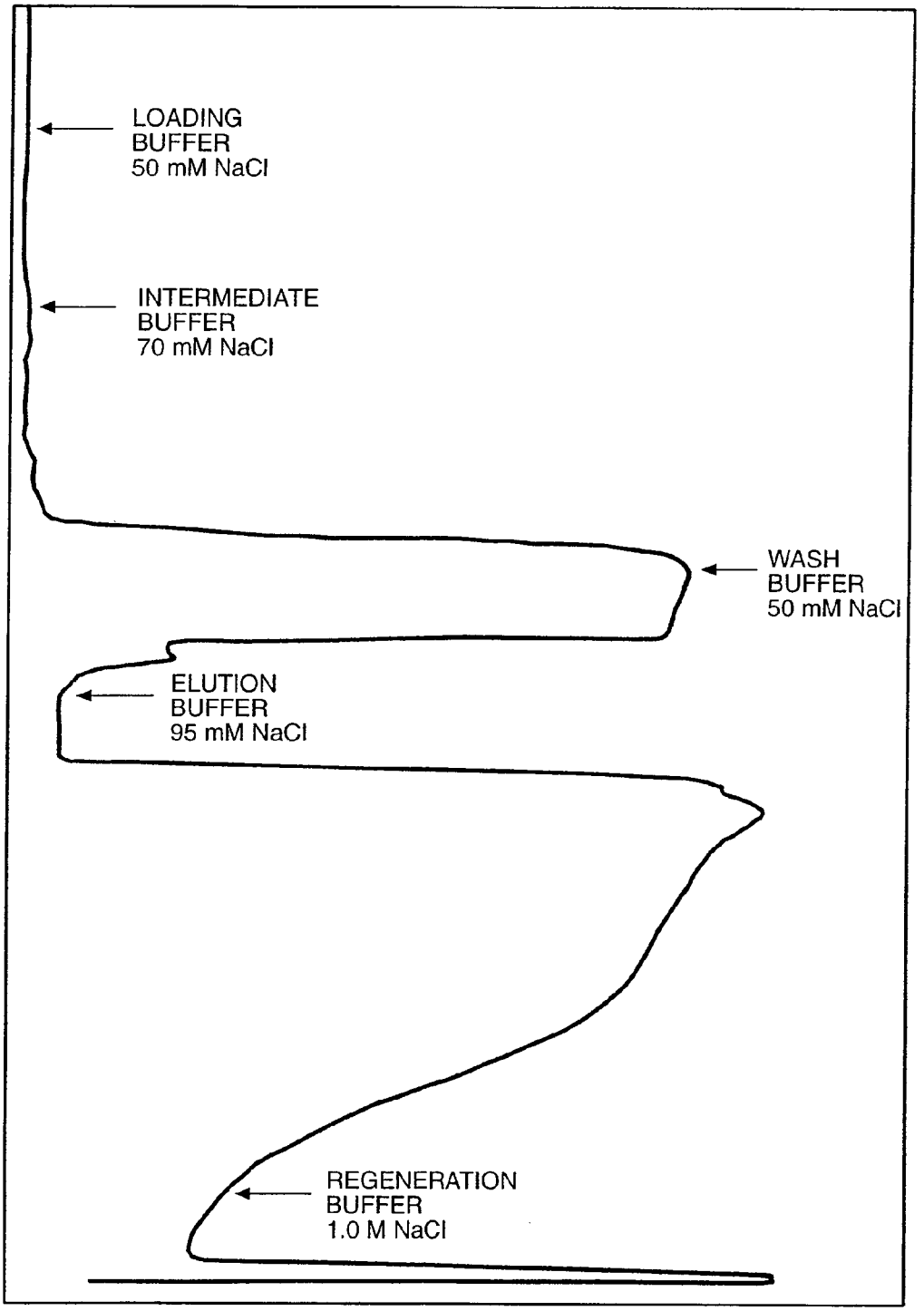
LOADING
BUFFER
50 mM NaCl
INTERMEDIATE
BUFFER
70 mM NaCl
WASH
BUFFER
50 mM NaCl
ELUTION
BUFFER
95 mM NaCl
REGENERATION
BUFFER
1.0 M NaCl

FIG._3

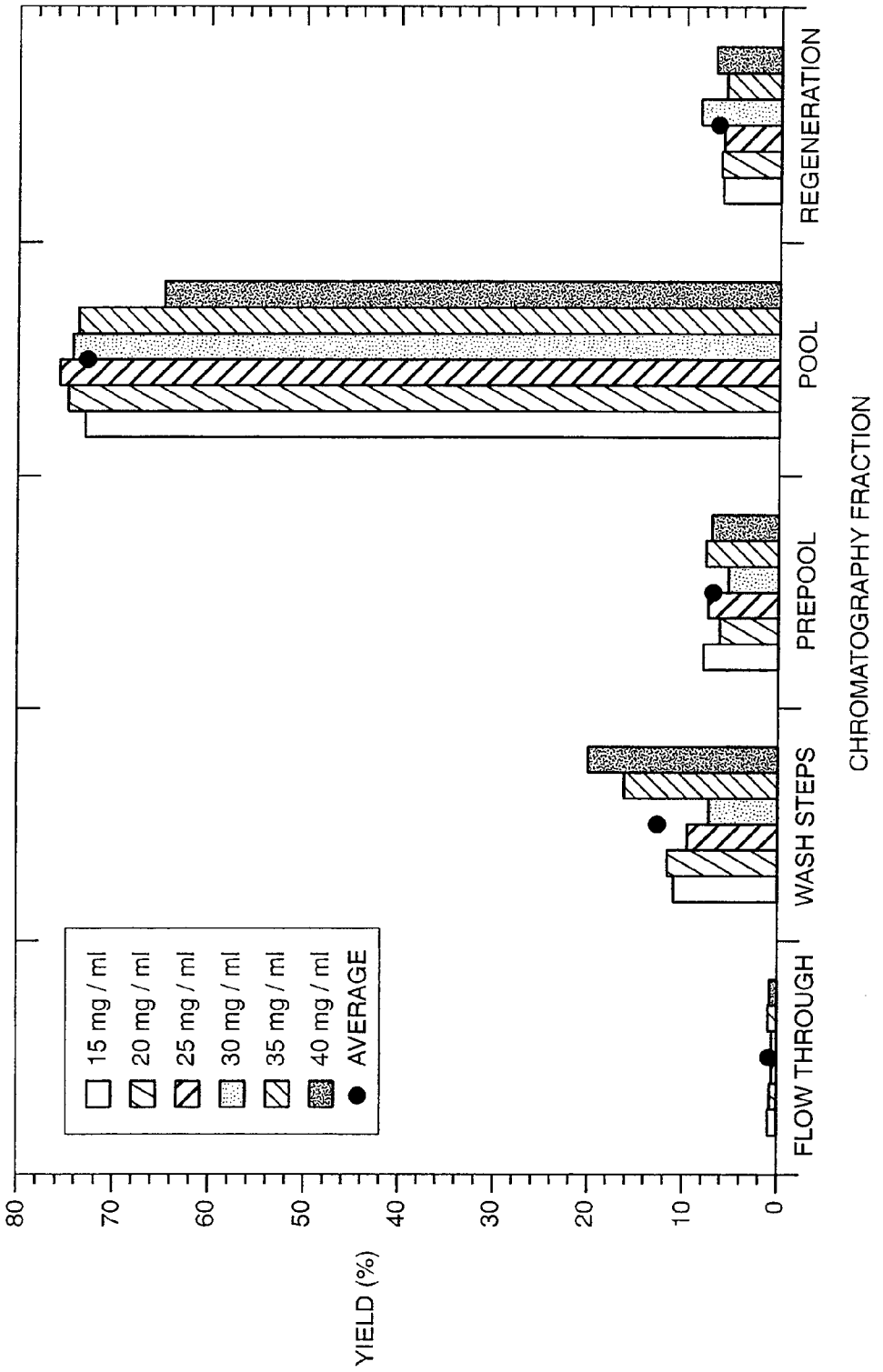
YIELD (%)
0
10
20
30
40
50
60
70
80
FLOW THROUGH
WASH STEPS
PREPOOL
POOL
REGENERATION
CHROMATOGRAPHY FRACTION
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
AVERAGE

FIG._4

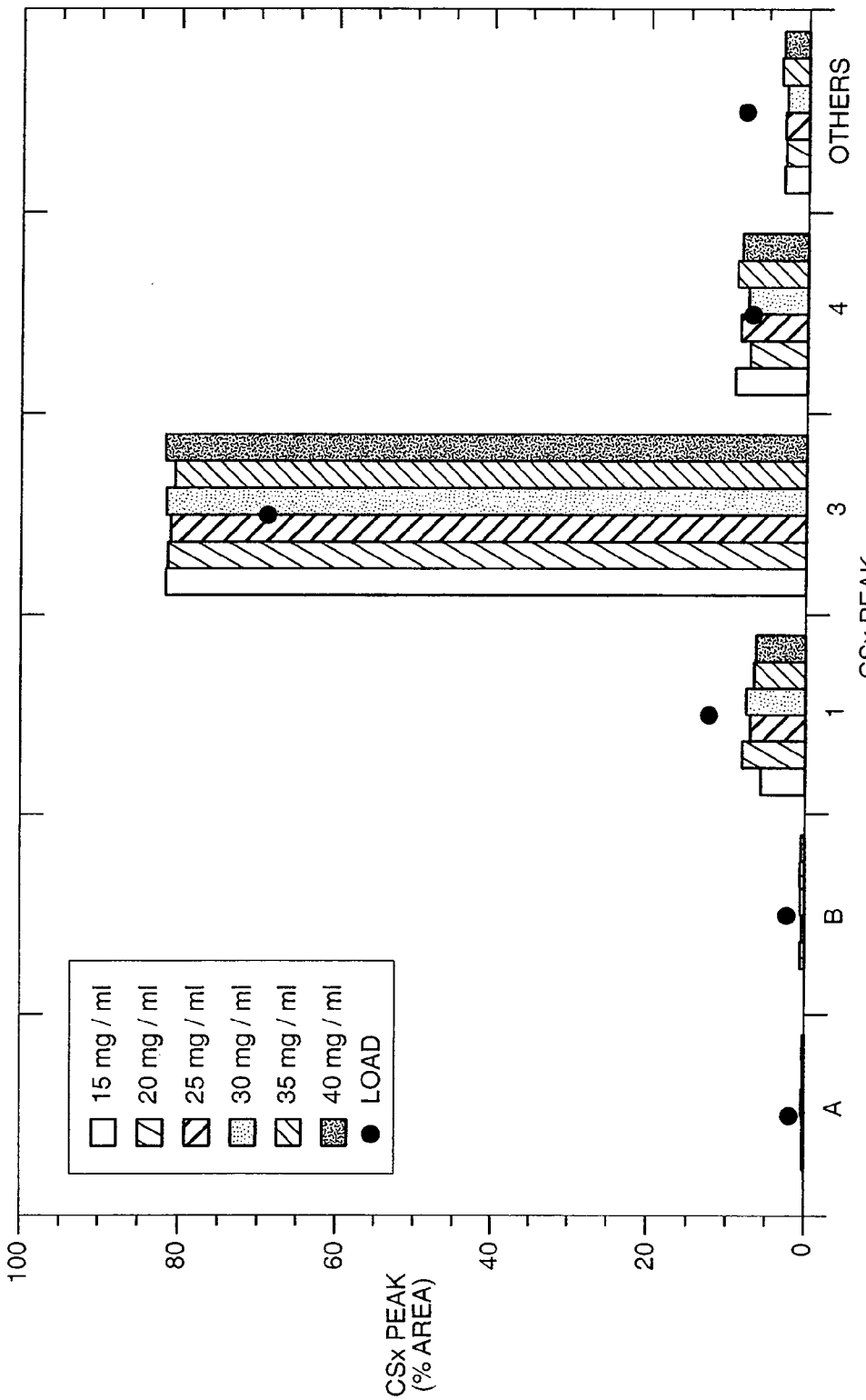
CSx PEAK (% AREA)
100
80
60
40
20
0
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
LOAD
A
B
1
3
4
OTHERS
CSx PEAK

FIG._5

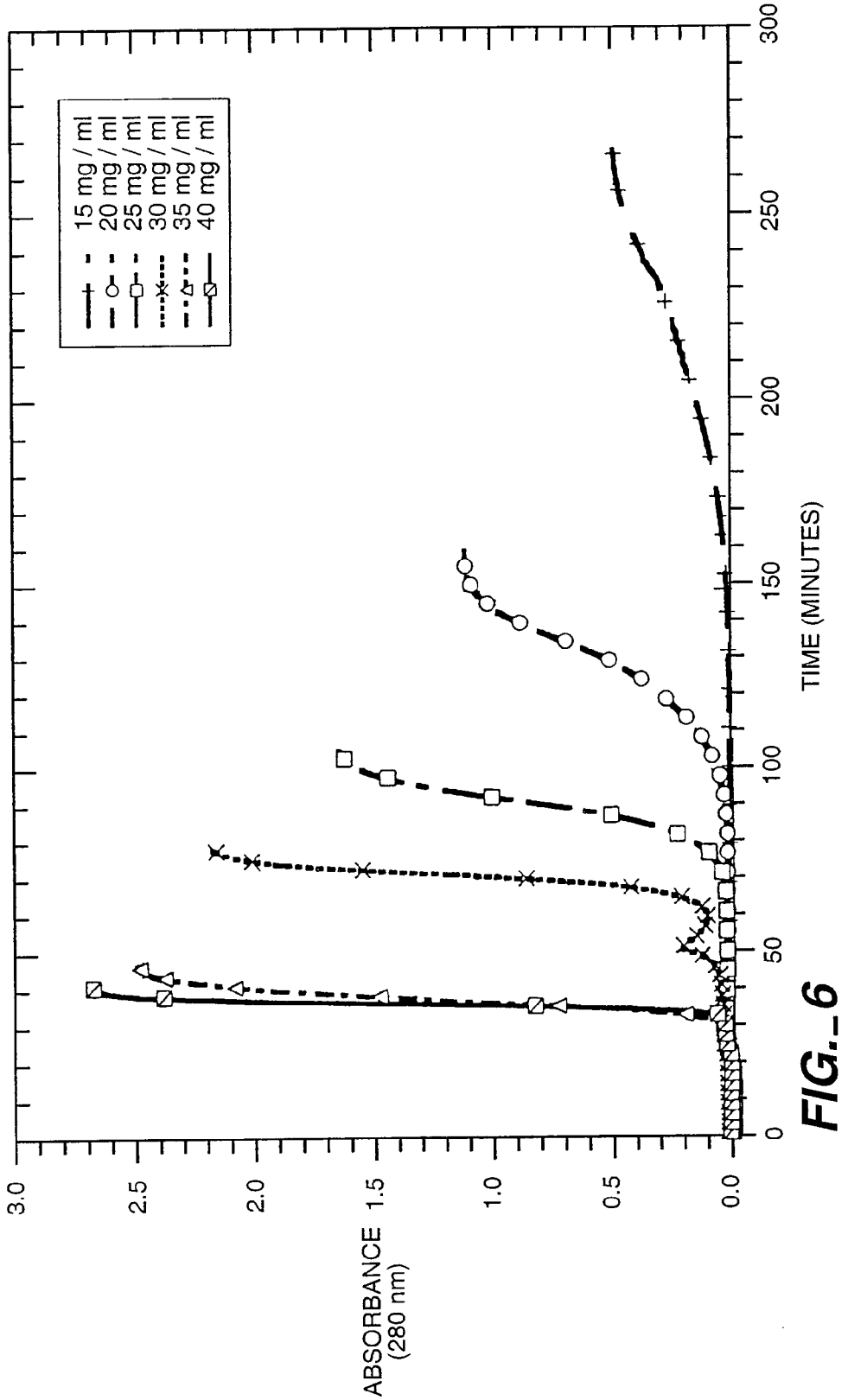
15 mg / ml
20 mg / ml
25 mg / ml
30 mg / ml
35 mg / ml
40 mg / ml
ABSORBANCE (280 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
TIME (MINUTES)

FIG._6

LIGHT CHAIN

```
1   D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q D V N T A V A W Y Q Q K P G K A P K
46  L L I Y S A S F L Y S G V P S R F S G S R S G T D F T L T I S S L Q P E D F A T Y Y C Q Q
91  H Y T T P P T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L
136 L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T
181 L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
```

15 30 45 60 75 90 105 120 135 150 165 180 195 210 214

FIG._7A

HEAVY CHAIN

```
1   E V Q L V E S G G G L V Q P G G S L R L S C A A S G F N I K D T Y I H W V R Q A P G K G L
46  E W V A R I Y P T N G Y T R Y A D S V K G R F T I S A D T S K N T A Y L Q M N S L R A E D
91  T A V Y Y C S R W G G D G F Y A M D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S
136 K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S
181 G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K
226 T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S
271 H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D
316 W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E
361 M T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G
406 S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
```

FIG._7B

PROTEIN PURIFICATION

This application is a continuation of U.S. application Ser. No. 12/418,905 filed Apr. 6, 2009 (now abandoned), which is a continuation of U.S. application Ser. No. 11/398,447, filed Apr. 5, 2006, (now U.S. Pat. No. 7,531,645 issued May 12, 2009) which is a continuation of and claims priority under 35 U.S.C. §120 to U.S. patent application Ser. No. 10/949,683, filed Sep. 24, 2004, (now U.S. Pat. No. 7,074,404 issued Jul. 11, 2006), which is a continuation of and which claims priority to under 35 USC §120 to U.S. application Ser. No. 10/253,366 filed Sep. 24, 2002 (now abandoned), which claims priority to U.S. application Ser. No. 09/304,465 filed May 3, 1999 (now U.S. Pat. No. 6,489,447 issued Dec. 3, 2002), which claims priority under 35 U.S.C. §119 to provisional application No. 60/084,459 filed May 6, 1998, the entire disclosures of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to protein purification. In particular, the invention relates to a method for purifying a polypeptide (e.g. an antibody) from a composition comprising the polypeptide and at least one contaminant using the method of ion exchange chromatography.

2. Description of Related Art

The large-scale, economic purification of proteins is increasingly an important problem for the biotechnology industry. Generally, proteins are produced by cell culture, using either mammalian or bacterial cell lines engineered to produce the protein of interest by insertion of a recombinant plasmid containing the gene for that protein. Since the cell lines used are living organisms, they must be fed with a complex growth medium, containing sugars, amino acids, and growth factors, usually supplied from preparations of animal serum. Separation of the desired protein from the mixture of compounds fed to the cells and from the by-products of the cells themselves to a purity sufficient for use as a human therapeutic poses a formidable challenge.

Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through".

Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins. In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; i.e., the pH or conductivity is increased or decreased in a single direction.

SUMMARY OF THE INVENTION

The present invention provides an ion exchange chromatographic method wherein a polypeptide of interest is bound to the ion exchange material at an initial conductivity or pH and then the ion exchange material is washed with an intermediate buffer at a different conductivity or pH, or both. At a specific point following this intermediate wash, and contrary to ion exchange chromatography standard practice, the ion exchange material is washed with a wash buffer where the change in conductivity or pH, or both, from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity or pH, or both, achieved in the previous steps. Only after washing with the wash buffer, is the ion exchange material prepared for the polypeptide molecule of interest to be eluted by the application of the elution buffer having a conductivity or pH, or both, which differ from the conductivity or pH, or both, of the buffers used in previous steps.

This novel approach to ion exchange chromatography is particularly useful in situations where a product molecule must be separated from a very closely related contaminant molecule at full manufacturing scale, where both purity and high recovery of polypeptide product are desired.

Accordingly, the invention provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to an ion exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the ion exchange material with an intermediate buffer at a second conductivity and/or pH so as to elute the contaminant from the ion exchange material;

(c) washing the ion exchange material with a wash buffer which is at a third conductivity and/or pH, wherein the change in conductivity and/or pH from the intermediate buffer to the wash buffer is in an opposite direction to the change in conductivity and/or pH from the loading buffer to the intermediate buffer; and

(d) washing the ion exchange material with an elution buffer at a fourth conductivity and/or pH so as to elute the

polypeptide from the ion exchange material. The first conductivity and/or pH may be the same as the third conductivity and/or pH.

Where the ion exchange material comprises a cation exchange resin, the conductivity and/or pH of the intermediate buffer is/are preferably greater than the conductivity and/or pH of the loading buffer; the conductivity and/or pH of the wash buffer is/are preferably less than the conductivity and/or pH of the intermediate buffer; and the conductivity and/or pH of the elution buffer is/are preferably greater than the conductivity and/or pH of the intermediate buffer. Preferably, the conductivity and/or pH of the wash buffer is/are about the same as the conductivity and/or pH of the loading buffer.

Preferably elution of the contaminant and of the polypeptide is achieved by modifying the conductivity of the intermediate buffer and of the elution buffer, respectively, while keeping the pH of these buffers approximately the same.

The invention also provides a method for purifying a polypeptide from a composition comprising the polypeptide and a contaminant, which method comprises the following steps performed sequentially:

(a) binding the polypeptide to a cation exchange material using a loading buffer, wherein the loading buffer is at a first conductivity and pH;

(b) washing the cation exchange material with an intermediate buffer at a second conductivity and/or pH which is greater than that of the loading buffer so as to elute the contaminant from the ion exchange material;

(c) washing the cation exchange material with a wash buffer which is at a third conductivity and/or pH which is less than that of the intermediate buffer; and

(d) washing the cation exchange material with an elution buffer at a fourth conductivity and/or pH which is greater than that of the intermediate-buffer so as to elute the polypeptide from the ion exchange material.

In addition, the invention provides a method for purifying an antibody from a composition comprising the antibody and a contaminant, which method comprises loading the composition onto a cation exchange resin, wherein the amount of antibody loaded onto the cation exchange resin is from about 20 mg to about 35 mg of the antibody per mL of cation exchange resin and, optionally, further comprising eluting the antibody from the cation exchange resin. The method preferably further comprises an intermediate wash step for eluting one or more contaminants from the ion exchange resin. This intermediate wash step usually precedes the step of eluting the antibody.

The invention further provides a composition comprising a mixture of anti-HER2 antibody and one or more acidic variants thereof, wherein the amount of the acidic variant(s) in the composition is less than about 25% and preferably less than about 20%, e.g. in the range from about 1% to about 18%. Optionally, the composition further comprises a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flow diagram showing how one could perform cation exchange chromatography by altering conductivity (e.g. to the NaCl concentrations of Example 1 below) or by altering pH (e.g. to the pH values as shown in the flow diagram).

FIG. **2** is a flow diagram showing how one could perform anion exchange chromatography by altering conductivity (e.g. to the NaCl concentrations as depicted in the figure) or by altering pH (e.g. to the pH values as shown).

FIG. **3** is an absorbance trace from a cation exchange chromatography run of Example 1 at full manufacturing scale. Points at which the column is washed with the different buffers described herein are marked with arrows.

FIG. **4** depicts recombinant humanized anti-HER2 monoclonal antibody (rhuMAb HER2) recovered in each chromatography fraction (calculated as the percentage of the sum total of all fractions of the relevant chromatography). Flow through, wash steps, and prepool fractions are all effluent samples collected from the onset of load to the initiation of pooling. The pool fraction is the five column volume effluent sample of elution starting at the leading shoulder's inflection point. The regeneration fraction contains effluent captured from the end of pooling to the end of regeneration.

FIG. **5** shows the quality of rhuMAb HER2 in each cation exchange chromatography pool sample as evaluated by carboxy sulfon cation exchange high pressure liquid chromatography (Csx HPIEX). Peaks A, B, and 1 are deamidated forms of rhuMAb HER2. Peak 3 is nondeamidated rhuMAb HER2. Peak 4 is a combination of C-terminal Lysine containing and iso-aspartate variants of rhuMAb HER2.

FIG. **6** shows the absorbance (280 nm) profiles of the 0.025 M MES/0.070 M NaCl, pH 5.6 wash for each chromatography. The mass of rhuMAb HER2 applied to the cation exchange resin effects the peak's absorbance level at the apex as well as the amount of buffer required to reach the apex. Due to minor peaks which occur (as best seen in the 30 mg/mL load) in this wash, the apex is defined as absorbance levels of at least 0.5 absorbance units (AU).

FIGS. **7**A and **7**B show the amino acid sequences of humMAb4D5-8 light chain (SEQ ID NO:1) and humMAb4D5-8 heavy chain (SEQ ID NO:2), respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The "composition" to be purified herein comprises the polypeptide of interest and one or more contaminants. The composition may be "partially purified" (i.e. having been subjected to one or more purification steps, such as Protein A Chromatography as in Example 1 below) or may be obtained directly from a host cell or organism producing the polypeptide (e.g. the composition may comprise harvested cell culture fluid).

As used herein, "polypeptide" refers generally to peptides and proteins having more than about ten amino acids. Preferably, the polypeptide is a mammalian protein, examples of which include renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-

lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins (IGFBPs); CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides. Most preferred is a full length antibody that binds human HER2.

A "contaminant" is a material that is different from the desired polypeptide product. The contaminant may be a variant of the desired polypeptide (e.g. a deamidated variant or an amino-aspartate variant of the desired polypeptide) or another polypeptide, nucleic acid, endotoxin etc.

A "variant" or "amino acid sequence variant" of a starting polypeptide is a polypeptide that comprises an amino acid sequence different from that of the starting polypeptide. Generally, a variant will possess at least 80% sequence identity, preferably at least 90% sequence identity, more preferably at least 95% sequence identity, and most preferably at least 98% sequence identity with the native polypeptide. Percentage sequence identity is determined, for example, by the Fitch et al., *Proc. Natl. Acad. Sci. USA* 80:1382-1386 (1983), version of the algorithm described by Needleman et al., *J. Mol. Biol.* 48:443-453 (1970), after aligning the sequences to provide for maximum homology. Amino acid sequence variants of a polypeptide may be prepared by introducing appropriate nucleotide changes into DNA encoding the polypeptide, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites. Methods for generating amino acid sequence variants of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

An "acidic variant" is a variant of a polypeptide of interest which is more acidic (e.g. as determined by cation exchange chromatography) than the polypeptide of interest. An example of an acidic variant is a deamidated variant.

A "deamidated" variant of a polypeptide molecule is a polypeptide wherein one or more asparagine residue(s) of the original polypeptide have been converted to aspartate, i.e. the neutral amide side chain has been converted to a residue with an overall acidic character. Deamidated humMAb4D5 antibody from the Example below has Asn30 in CDR1 of either or both of the V_L regions thereof converted to aspartate. The term "deamidated human DNase" as used herein means human DNase that is deamidated at the asparagine residue that occurs at position 74 in the amino acid sequence of native mature human DNase (U.S. Pat. No. 5,279,823; expressly incorporated herein by reference).

The term "mixture" as used herein in reference to a composition comprising an anti-HER2 antibody, means the presence of both the desired anti-HER2 antibody and one or more acidic variants thereof.

The acidic variants may comprise predominantly deamidated anti-HER2 antibody, with minor amounts of other acidic variant(s). It has been found, for example, that in preparations of anti-HER2 antibody obtained from recombinant expression, as much as about 25% of the anti-HER2 antibody is deamidated.

In preferred embodiments of the invention, the polypeptide is a recombinant polypeptide. A "recombinant polypeptide" is one which has been produced in a host cell which has been transformed or transfected with nucleic acid encoding the polypeptide, or produces the polypeptide as a result of homologous recombination. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing nucleic acid into a cell. Following transformation or transfection, the nucleic acid may integrate into the host cell genome, or may exist as an extrachromosomal element. The "host cell" includes a cell in in vitro cell culture as well a cell within a host animal. Methods for recombinant production of polypeptides are described in U.S. Pat. No. 5,534,615; expressly incorporated herein by reference, for example.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

The antibody herein is directed against an "antigen" of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those polypeptides discussed above. Preferred molecular targets for antibodies encompassed by the present invention include CD polypeptides such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and av/b3 integrin including either a or b subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mp1 receptor; CTLA-4; polypeptide C etc. Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may

be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). In a further embodiment, "monoclonal antibodies" can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Polypeptides of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The CDR and FR residues of the rhuMAb HER2 antibody of the example below (humAb4D5-8) are identified in Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these dis-

plays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185. "Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata et al. *Polypeptide Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

"Multispecific antibodies" have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-$p185^{HER2}$/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-$p185^{HER2}$, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell ahesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. FcγRI, or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-$p185^{HER2}$/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal

ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

The phrase "ion exchange material" refers to a solid phase which is negatively charged (i.e. a cation exchange resin) or positively charged (i.e. an anion exchange resin). The charge may be provided by attaching one or more charged ligands to the solid phase, e.g. by covalent linking. Alternatively, or in addition, the charge may be an inherent property of the solid phase (e.g. as is the case for silica, which has an overall negative charge).

By "solid phase" is meant a non-aqueous matrix to which one or more charged ligands can adhere. The solid phase may be a purification column, a discontinuous phase of discrete particles, a membrane, or filter etc. Examples of materials for forming the solid phase include polysaccharides (such as agarose and cellulose); and other mechanically stable matrices such as silica (e.g. controlled pore glass), poly(styrenedivinyl)benzene, polyacrylamide, ceramic particles and derivatives of any of the above.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, BAKERBOND ABX™, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from Pharmacia) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from Pharmacia).

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (Pharmacia).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the

desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., Ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 5 to about 7 (e.g. as in Example 1 below). Examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more contaminants onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more contaminants) is/are bound to the ion exchange resin.

The "intermediate buffer" is used to elute one or more contaminants from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that the contaminant is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all contaminants and the polypeptide of interest from the ion exchange resin.

The term "conductivity" refers to the ability of an aqueous solution to conduct an electric current between two electrodes. In solution, the current flows by ion transport. Therefore, with an increasing amount of ions present in the aqueous solution, the solution will have a higher conductivity. The unit of measurement for conductivity is mmhos (mS/cm), and can be measured using a conductivity meter sold, e.g., by Orion. The conductivity of a solution may be altered by changing the concentration of ions therein. For example, the concentration of a buffering agent and/or concentration of a salt (e.g. NaCl or KCl) in the solution may be altered in order to achieve the desired conductivity. Preferably, the salt concentration of the various buffers is modified to achieve the desired conductivity as in the Example below.

By "purifying" a polypeptide from a composition comprising the polypeptide and one or more contaminants is meant increasing the degree of purity of the polypeptide in the composition by removing (completely or partially) at least one contaminant from the composition. A "purification step" may be part of an overall purification process resulting in a "homogeneous" composition, which is used herein to refer to a composition comprising at least about 70% by weight of the polypeptide of interest, based on total weight of the composition, preferably at least about 80% by weight.

Unless indicated otherwise, the term "HER2" when used herein refers to human HER2 protein and "HER2" refers to human HER2 gene. The human HER2 gene and HER2 protein are described in Semba et al., *PANAS* (*USA*) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363), for example.

The term "humMAb4D5-8" when used herein refers to a humanized anti-HER2 antibody comprising the light chain amino acid sequence of SEQ ID NO:1 and the heavy chain amino acid sequence of SEQ ID NO:2 or amino acid sequence variants thereof which retain the ability to bind HER2 and inhibit growth of tumor cells which overexpress HER2 (see U.S. Pat. No. 5,677,171; expressly incorporated herein by reference).

The "pI" or "isoelectric point" of a polypeptide refer to the pH at which the polypeptide's positive charge balances its negative charge. pI can be calculated from the net charge of the amino acid residues of the polypeptide or can be determined by isoelectric focussing (e.g. using CSx chromatography as in the Example below).

By "binding" a molecule to an ion exchange material is meant exposing the molecule to the ion exchange material under appropriate conditions (pH/conductivity) such that the molecule is reversibly immobilized in or on the ion exchange material by virtue of ionic interactions between the molecule and a charged group or charged groups of the ion exchange material.

By "washing" the ion exchange material is meant passing an appropriate buffer through or over the ion exchange material.

To "elute" a molecule (e.g. polypeptide or contaminant) from an ion exchange material is meant to remove the molecule therefrom by altering the ionic strength of the buffer surrounding the ion exchange material such that the buffer competes with the molecule for the charged sites on the ion exchange material.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with the polypeptide purified as described herein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the polypeptide. The label may be itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL™, Bristol-Myers Squibb Oncology, Princeton, N.J.), and doxetaxel, toxotere, methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, caminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

MODES FOR CARRYING OUT THE INVENTION

The invention herein provides a method for purifying a polypeptide from a composition (e.g. an aqueous solution)

comprising the polypeptide and one or more contaminants. The composition is generally one resulting from the recombinant production of the polypeptide, but may be that resulting from production of the polypeptide by peptide synthesis (or other synthetic means) or the polypeptide may be purified from a native source of the polypeptide. Preferably the polypeptide is an antibody, e.g. one which binds the HER2 antigen.

For recombinant production of the polypeptide, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide is readily isolated and sequenced using conventional procedures (e.g., where the polypeptide is an antibody by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (e.g. as described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella*, e.g., *Salmonella typhimurium, Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 Apr. 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe; Kluyveromyces* hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183, 070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa; Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

Suitable host cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/−DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (e.g. resulting from homogenization), is removed, for example, by centrifugation or ultrafiltration. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

The polypeptide is then subjected to one or more purification steps, including the ion exchange chromatography method as claimed herein. Examples of additional purifica-

tion procedures which may be performed prior to, during, or following the ion exchange chromatography method include fractionation on a hydrophobic interaction chromatography (e.g. on phenyl sepharose), ethanol precipitation, isoelectric focusing, Reverse Phase HPLC, chromatography on silica, chromatography on HEPARIN SEPHAROSE™, further anion exchange chromatography and/or further cation exchange chromatography, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography (e.g. using protein A, protein G, an antibody, a specific substrate, ligand or antigen as the capture reagent).

Ion exchange chromatography is performed as claimed herein. A decision is first made as to whether an anion or cation exchange resin is to be employed. In general, a cation exchange resin may be used for polypeptides with pIs greater than about 7 and an anion exchange resin may be used for polypeptides with pIs less than about 7.

The anion or cation exchange resin is prepared according to known methods. Usually, an equilibration buffer is passed through the ion exchange resin prior to loading the composition comprising the polypeptide and one or more contaminants onto the resin. Conveniently, the equilibration buffer is the same as the loading buffer, but this is not required.

The various buffers used for the chromatography depend, for example, on whether a cation or anion exchange resin is employed. This is shown more clearly in the flow diagrams of FIGS. **1** and **2**.

With particular reference to FIG. **1**, which shows exemplary steps to be performed where a cation exchange resin is used, the pH and/or conductivity of each buffer is/are increased relative to the preceding buffer, except for the wash buffer where the conductivity and/or pH is/are less than the conductivity and/or pH of the preceding intermediate buffer. The aqueous solution comprising the polypeptide of interest and contaminant(s) is loaded onto the cation exchange resin using the loading buffer that is at a pH and/or conductivity such that the polypeptide and the contaminant bind to the cation exchange resin. As in the Example below, the loading buffer may be at a first low conductivity (e.g. from about 5.2 to about 6.6 mmhos). An exemplary pH for the loading buffer may be about 5.0 (see FIG. **1**). From about 20 mg/mL to about 35 mg/mL of the polypeptide (e.g. of a full length antibody) may, for example, be loaded on the ion exchange resin.

The cation exchange resin is then washed with an intermediate buffer which is at a second conductivity and/or pH so as to essentially elute the contaminant, but not a substantial amount of the polypeptide of interest. This may be achieved by increasing the conductivity or pH, or both, of the intermediate buffer. The change from loading buffer to intermediate buffer may be step-wise or gradual as desired. In the Example herein, the intermediate buffer had a greater conductivity than that of the loading buffer (i.e. the intermediate buffer's conductivity was in the range from about 7.3 to about 8.4 mmhos). Alternatively, as shown in FIG. **1**, the pH of the intermediate buffer may exceed that of the loading buffer in this embodiment of the invention, where a cation exchange resin is used. For example, the intermediate buffer may have a pH of about 5.4.

Following washing with the intermediate buffer, the cation exchange resin is washed or re-equilibrated with the wash buffer which has a conductivity or pH, or both, which is/are less than that of the intermediate buffer (i.e. the conductivity, or pH, or both, is/are changed in an opposite, i.e. reverse, direction to the preceding step, unlike ion exchange chromatography steps in the literature). In the Example below, the wash buffer had about the same conductivity as the loading buffer (i.e. in the range from about 5.2 to about 6.6 mmhos) and its conductivity was, therefore, less than that of the intermediate buffer. In another embodiment, one may reduce the conductivity of the wash buffer to a conductivity that is less than, or greater than, that of the loading buffer, provided the conductivity of the wash buffer is less than that of the intermediate buffer. In another embodiment, the pH of the wash buffer may be less than the pH of the intermediate buffer (e.g. the pH of the wash buffer may about 5.0). The change in conductivity and/or pH of the wash buffer compared to the intermediate buffer may be achieved by step-wise or gradual change of either or both of these parameters.

After the wash step of the preceding paragraph, the cation exchange resin is prepared for elution of the desired polypeptide molecule therefrom. This is achieved using an elution buffer that has a pH and/or conductivity such that the desired polypeptide no longer binds to the cation exchange resin and therefore is eluted therefrom. The pH and/or conductivity of the elution buffer generally exceed(s) the pH and/or conductivity of the loading buffer, the intermediate buffer and the wash buffer used in the previous steps. In the Example below, the conductivity of the elution buffer was in the range from about 10.0 to about 11.0 mmhos. Alternatively, or in addition, the pH of the elution buffer may be increased relative to the wash buffer and to the intermediate buffer (for example, the pH of the elution buffer may about 6.0). The change in conductivity and/or pH may be step-wise or gradual, as desired. Hence, the desired polypeptide is retrieved from the cation exchange resin at this stage in the method.

In an alternative embodiment, the ion exchange material comprises an anion exchange resin. This embodiment of the invention is depicted in FIG. **2** herein. As illustrated in this figure, the changes in conductivity are generally as described above with respect to a cation exchange resin. However, the direction of change in pH is different for an anion exchange resin. For example, if elution of the contaminant(s) and polypeptide are to be achieved by altering pH, the loading buffer has a first pH and the pH is decreased in the intermediate buffer so as to elute the contaminant or contaminants. In the third step, the column is washed/re-equilibrated with the wash buffer and the change in conductivity or pH, or both, is in the opposite direction to that of the previous step. Hence, the pH may be increased in the wash buffer, compared to the intermediate buffer. Following this step, the polypeptide of interest is eluted from the anion exchange resin using an elution buffer at a fourth conductivity and/or pH. If pH is altered, it will normally be less than the pH of the loading buffer, the intermediate buffer and the wash buffer. The change in pH and/or conductivity in progressive buffers can, as explained above, be step-wise or gradual.

In the preferred embodiment of the invention, a single parameter (i.e. either conductivity or pH) is changed to achieve elution of both the polypeptide and contaminant, while the other parameter (i.e. pH or conductivity, respectively) remains about constant. For example, while the conductivity of the various buffers (loading buffer, intermediate buffer, wash buffer and/or elution buffer) may differ, the pH's thereof may be essentially the same.

In an optional embodiment of the invention, the ion exchange resin is regenerated with a regeneration buffer after elution of the polypeptide, such that the column can be re-used. Generally, the conductivity and/or pH of the regeneration buffer is/are such that substantially all contaminants and the polypeptide of interest are eluted from the ion exchange resin. Generally, the regeneration buffer has a very high conductivity for eluting contaminants and polypeptide from the ion exchange resin.

The method herein is particularly useful for resolving a polypeptide molecule of interest from at least one contaminant, where the contaminant and polypeptide molecule of interest differ only slightly in ionic charge. For example, the pIs of the polypeptide and contaminant may be only "slightly different", for example they may differ by only about 0.05 to about 0.2 pI units. In the Example below, this method could be used to resolve an anti-HER2 antibody having a pI of 8.87, from a singly-deamidated variant thereof having a pI of 8.79. Alternatively, the method may be used to resolve a deamidated DNase, for example, from nondeamidated DNase. In another embodiment, the method may be used to resolve a polypeptide from a glycosylation variant thereof, e.g. for resolving a variant of a polypeptide having a different distribution of sialic acid compared to the nonvariant polypeptide.

The polypeptide preparation obtained according to the ion exchange chromatography method herein may be subjected to additional purification steps, if necessary. Exemplary further purification steps have been discussed above.

Optionally, the polypeptide is conjugated to one or more heterologous molecules as desired. The heterologous molecule may, for example, be one which increases the serum half-life of the polypeptide (e.g. polyethylene glycol, PEG), or it may be a label (e.g. an enzyme, fluorescent label and/or radionuclide) or a cytotoxic molecule (e.g. a toxin, chemotherapeutic drug, or radioactive isotope etc).

A therapeutic formulation comprising the polypeptide, optionally conjugated with a heterologous molecule, may be prepared by mixing the polypeptide having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. "Pharmaceutically acceptable" carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). The humMAb4D5-8 antibody of particular interest herein may be prepared as a lyophilized formulation, e.g. as described in WO 97/04801; expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. For example, for an anti-HER2 antibody a chemotherapeutic agent, such as a taxoid or tamoxifen, may be added to the formulation.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulation to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide variant, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid.

The polypeptide purified as disclosed herein or the composition comprising the polypeptide and a pharmaceutically acceptable carrier is then used for various diagnostic, therapeutic or other uses known for such polypeptides and compositions. For example, the polypeptide may be used to treat a disorder in a mammal by administering therapeutically effective amount of the polypeptide to the mammal.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

Example 1

Full length human IgG rhuMAb HER2 (humAb4D5-8 in Carter et al. *Proc. Natl. Acad. Sci.* 89: 4285-4289 (1992) comprising the light chain amino acid sequence of SEQ ID NO:1 and heavy chain amino acid sequence of SEQ ID NO:2) was produced recombinantly in CHO cells. Following protein production and secretion to the cell culture medium, the CHO cells were separated from the cell culture medium by tangential flow filtration (PROSTACK™). Protein A chromatography was then performed by applying the Harvested Cell Culture Fluid (HCCF) from the CHO cells directly to an equilibrated PROSEP A™ column (Bioprocessing, Ltd).

Following Protein A chromatography, cation exchange chromatography was performed using a sulphopropyl (SP)-SEPHAROSE FAST FLOW™ (SPSFF) column (Pharmacia) to further separate the desired anti-HER2 antibody molecule. The chromatography operation was performed in bind and elute mode.

The SPSFF column was prepared for load by sequential washes with regeneration buffer (0.025 M MES/1.0 M NaCl, pH 5.6) followed by equilibration buffer (0.025 M MES/50 mM NaCl, pH 5.6). The column was then loaded with Protein A pool adjusted to a pH of 5.60±0.05 and a conductivity of 5.8±0.2 mmhos. Prior to elution, the column was washed in three steps: (1) loading buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1 column volume; (2) intermediate buffer (0.025 M MES/70 mM NaCl, pH 5.6) until an apex of a 280 nm peak was reached; and (3) wash buffer (0.025 M MES/50 mM NaCl, pH 5.6) for a minimum of 1.2 column volumes. rhuMAb HER2 was then eluted from the column

with elution buffer (0.025 M MES/95 mM NaCl, pH 5.6). The elution 280 nm profile has a shoulder on the leading edge (FIG. **3**). At the inflection point of this shoulder, pooling starts and continues for an additional 5 column volumes. The column was then regenerated with regeneration buffer (0.025 M MES/1.0 M NaCl, pH 5.6).

Materials and Methods

Column and Load Preparation:

A reduced-scale SPSFF column was packed. The dimensions were: 27.0 mL volume, 1.0 cm diameter and 34.5 cm bed height. The pH of an aliquot of Protein A pool was titered to 5.6 with 1.5 M Tris base. The conductivity of the pool was reduced by the addition of an equal volume of sterile water for injection (SWFI).

Chromatography:

The chromatography runs for this study were performed with Pharmacia's UNICORN™ FPLC system. The equilibration, load, and initial wash steps were performed at a linear flow rate of 200 cm/h. All chromatography steps were performed at a linear flow rate of 100 cm/h. The sequence of chromatography steps are defined in Table 1. A total of six chromatography runs were performed with load densities of 15, 20, 25, 30, 35, and 40 mg of rhuMAb HER2 per mL of SPSFF resin.

TABLE 1

Chromatography Steps[1]

Chromatography Step	Buffer	Approximate Endpoint
Equilibration: Part 1	0.025M MES/1.0M NaCl, pH 5.6	2 CV[2]
Equilibration: Part 2	0.025M MES/0.05M NaCl, pH 5.6	pH: 5.6 ± 0.1 Cond.: 5.8 ± 0.2 mmhos
Load	Adjusted Protein A Pool	As Required
Wash 1	0.025M MES/0.05M NaCl, pH 5.6	1.5 CV
Wash 2	0.025M MES/0.07M NaCl, pH 5.6	Apex of Peak
Wash 3	0.025M MES/0.05M NaCl, pH 5.6	2 CV
Elution: Prepool	0.025M MES/0.095M NaCl, pH 5.6	To Leading Shoulder's Inflection Point (−1.2 CV)
Elution: Pool	0.025M MES/0.095M NaCl, pH 5.6	5 CV
Regeneration	0.025M MES/1.0M NaCl, pH 5.6	2 CV

[1]The equilibration of the resin was performed in manual mode; the remaining steps were executed from a Pharmacia Unicorn Program.
[2]CV = column volume(s).

Total Protein:

The protein concentration of each chromatography fraction (flow through, wash steps, elution prepool, elution pool, and regeneration) was determined by spectrophometric scans of each sample. The results were used to calculate product recovery yields. The extinction coefficient for rhuMAb HER2 is 1.45. Calculations used to derive the results (FIG. **4**) are:

$$\text{Protein Concentration (mg/mL)} = \frac{280\text{ nm}}{1.45} \times \text{Dilution Factor}$$

$$\text{Protein Mass (mg) in Each Fraction} = \text{Protein Concentration (mg/mL)} \times \text{Fraction Volume (mL)}$$

$$\text{Yield (\%)} = \frac{\text{Fraction Mass (mg)}}{\text{Total Mass (mg)}} \times 100$$

Determination of rhuMAb HER2 Antibody Variants (CSx HPIEX):

The rhuMAb HER2 SPSFF chromatography column resolves antibody variants. Fractions from each of the study chromatographies were tested for the relative amount of variant antibody by CSx HPIEX chromatography. A BAKERBOND WIDE-PORE™ CSx HPIEX column (4.6×250 mm) was run at 1 mL/min at 55° C. The mobile phase was formed from a tertiary gradient (Table 2).

TABLE 2

Gradient Scheme

Time (min)	% A	% B	% C
0 - Initial Conditions	49	1	50
10.0	40	10	50
50.0	33	17	50
50.2	49	1	50
70.0	49	1	50

The column is run at 1 mL/min at 55° C.

The A buffer was 0.025 M MES, pH 5.9; the B buffer was 1 M Ammonium Acetate, pH 7.0; and the C solution was sterile water for injection. The column was equilibrated with the gradient's initial conditions (49% A; 1% B; and 50% C) and 200 µl of sample, diluted with SWFI and containing <300 µg protein, was injected. Each resulting chromatogram was integrated to determine the percent area of each peak for each fraction (Table 3 and FIG. **5**).

TABLE 3

CSx HPIEX analysis of rhuMAb HER2

CSx Peak	rhuMAb HER2 Variant
A & B	Light Chain: Asn →Asp[30] deamidation - and - Other unidentifiable variation by tryptic map
1	Light Chain: Asn → Asp[30] deamidation
3	Fully Processed Antibody
4	Heavy Chain: Asp → Iso-Asp[102] - and/or - Heavy Chain: An Additional Lys[450]
Others	Heavy Chain: Asp → Succinimide[102] - and/or - Multiple permutations found in Peaks 1 and 4

Chromatograms Compared:

The absorbance data (AU 280 nm) from each chromatography file was exported from Unicorn in ASCII format. The data from the 0.025 M MES/0.07 M NaCl, pH 5.6 wash was translated into Excel format and copied into KALEIDAGRAPH™. Using KALEIDAGRAPH™, the wash profiles were overlaid (FIG. **6**) and compared to each other.

Results and Discussion

Deamidated and other acidic variants of rhuMAb HER2 were produced when the antibody was made by recombinant DNA technology (see e.g., CSx peaks a, b and 1 in FIG. **5**). The deamidated and other acidic variants constituted about 25% (calculated as area under the integrated curve or profile obtained by CSx chromatography) of the composition obtained from the initial Protein A chromatography step. It was discovered that the ion exchange method described herein could be used to substantially reduce the amount of deamidated and other acidic variants in the anti-HER2 composition, i.e. to about 13% or less (i.e. the amount of acidic

variants in the preparation subjected to cation exchange chromatography as described herein was decreased by about 50% or more).

An absorbance trace from a cation exchange column run performed as described above is shown in FIG. **3**. This method resolved a deamidated variant of anti-HER2 antibody that differed only slightly from nondeamidated anti-HER2 antibody. The increase in conductivity from the initial conditions to the intermediate wash began to elute the deamidated anti-HER2 antibody. However, continued washing at this conductivity was found to elute nondeamidated anti-HER2 antibody, resulting in a loss of product. Proceeding directly from the intermediate buffer to the elution buffer was observed to result in either an unacceptably low removal of deamidated anti-HER2 antibody from the product if pooling began early or unacceptably low yields of anti-HER2 antibody product if pooling was delayed until the deamidated anti-HER2 antibody was reduced. It was discovered that by going back to lower conductivity as used initially, the elution of deamidated anti-HER2 antibody continued, without significant anti-HER2 antibody product elution.

The effect of rhuMAb HER2 load on (a) buffer requirements, (b) product recovery in the pool, and (c) product quality in the pool was evaluated.

At load densities of 15 mg/mL up to 35 mg/mL, the product yield in the elution pool is approximately 75%. For the load density of 40 mg/mL, the product yield in the pool dropped to 65% (FIG. **4**). This reduced recovery in the pool is largely attributed to an increased antibody in the two wash steps (at 70 mM NaCl and 50 mM NaCl, respectively).

The quality of rhuMAb HER2 in all the elution pools is equivalent as determined by CSx HPIEX analysis (FIG. **5**). Compared to the load material; there is an enrichment of the nondeamidated antibody (Peak 3), no change in the amount Iso-Asp^{102} or Lys^{450} antibody (Peak 4), and a reduction of the amount of Asp^{30} deamidated antibody (Peaks a, b, 1 and others).

The quality of rhuMAb HER2 in these cation pools is improved through the intermediate wash step. As the mass of rhuMAb HER2 bound to the resin increases, the intermediate buffer volume consumption needed to reach the apex of the 280 nm peak decreases. The buffer volume required for a 40 mg/mL load density is approximately 2.5 column volumes. The buffer volume required for a 15 mg/mL load density is approximately 15 column volumes. The exact increase of buffer requirement is not linear with the 5 mg/mL incremental changes between these two extremes. The greatest increase is seen between the load densities of 20 mg/mL and 15 mg/mL. Here the requirement doubles from 7.5 column volumes to the previously mentioned 15 column volumes of buffer. If the apex of the 70 mM NaCl wash peak is reached, however, the product quality is equivalent for any of load densities examined.

This study determined how much rhuMAb HER2 can be loaded onto the SPSFF resin. Between the ranges of 15 to 40 mg of antibody per mL of resin, there is no difference in the quality of rhuMAb HER2 recovered in the elution pool. The quantity of rhuMAb HER2 recovered, however, is reduced by approximately 10% when the resin is loaded with greater than 35 mg/mL. For consistent yields it is recommended that 35 mg/mL be set as the maximum load for manufacture of rhuMAb HER2. Furthermore, due to the substantial increase in the 70 mM NaCl wash volume requirement between the 20 and 15 mg/mL; it is recommended that 20 mg/mL be set as the minimal load for manufacture of rhuMAb HER2.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 2

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<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 1

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  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                 20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
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-continued

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                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys


<210> SEQ ID NO 2
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<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                95                  100                 105

Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                 120

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser
                125                 130                 135

Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys
                140                 145                 150

Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
                155                 160                 165

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
                170                 175                 180

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
                185                 190                 195

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
                200                 205                 210

Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
                215                 220                 225

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                230                 235                 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255
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-continued

```
Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
                260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
                275                 280                 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
                290                 295                 300

Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
                305                 310                 315

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
                320                 325                 330

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
                335                 340                 345

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
                350                 355                 360

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
                365                 370                 375

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
                380                 385                 390

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
                395                 400                 405

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
                410                 415                 420

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
                425                 430                 435

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                440                 445
```

The invention claimed is:

1. A therapeutic composition comprising a mixture of anti-HER2 antibody and one or more acidic variants thereof,

wherein the amount of the acidic variant(s) is less than about 25%,

and wherein the acidic variant(s) are predominantly deamidated variants wherein one or more asparagine residues of the anti-HER2 antibody have been deamidated,

and wherein the anti-HER2 antibody is humMAb4D5-8,

and wherein the deamidated variants have Asn30 in CDR1 of either or both V_L regions of humMAb4D5-8 converted to aspartate,

and a pharmaceutically acceptable carrier.

2. The therapeutic composition of claim **1**, wherein the amount of the acidic variant(s) is less than about 20%.

3. The therapeutic composition of claim **2**, wherein the amount of the acidic variant(s) is less than about 13%.

4. The therapeutic composition of claim **2**, wherein the amount of the acidic variant(s) is in the range of about 1 to 18%.

5. The therapeutic composition of any one of claims **1** to **4**, wherein the anti-HER2 antibody comprises the light chain amino acid sequence of SEQ ID NO 1 and the heavy chain amino acid sequence of SEQ ID NO: 2.

6. The therapeutic composition of any one of claims **1** to **4**, which is in the form of a lyophilized formulation or an aqueous solution.

7. The therapeutic composition of claim **5**, which is in the form of a lyophilized formulation or an aqueous solution.

* * * * *

EXHIBIT L

US007501122B2

(12) **United States Patent**
Adams et al.

(10) **Patent No.: US 7,501,122 B2**
(45) **Date of Patent: Mar. 10, 2009**

(54) **TREATMENT WITH ANTI-ERBB2 ANTIBODY COMBINATIONS**

(75) Inventors: **Camellia W. Adams**, Mountain View, CA (US); **Leonard G. Presta**, San Francisco, CA (US); **Mark Sliwkowski**, San Carlos, CA (US)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 192 days.

(21) Appl. No.: **11/223,361**

(22) Filed: **Sep. 9, 2005**

(65) **Prior Publication Data**

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Related U.S. Application Data

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(51) **Int. Cl.**
A61K 39/395 (2006.01)
C07K 16/30 (2006.01)
C07K 16/28 (2006.01)

(52) **U.S. Cl.** **424/143.1**; 424/130.1; 424/133.1; 424/135.1; 424/155.1; 424/156.1; 424/174.1

(58) **Field of Classification Search** 424/130.1, 424/133.1, 135.1, 143.1, 155.1, 156.1, 174.1
See application file for complete search history.

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(57) **ABSTRACT**

The present application describes methods for treating ErbB-expressing cancer with anti-ErbB2 antibody combinations.

9 Claims, 14 Drawing Sheets

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  1 MELAALCRWG LLLALLPPGA ASTQVCTGTD MKLRLPASPE THLDMLRHLY QGCQVVQGNL ELTYLPTNAS LSFLQDIQEV QGYVLIAHNQ VRQVPLQRLR

101 IVRGTQLFED NYALAVLDNG DPLNNTTPVT GASPGGLREL QLRSLTEILK GGVLIQRNPQ LCYQDTILWK DIFHKNNQLA LTLIDTNRSR ACHPCSPMCK

201 GSRCWGESSE DCQSLTRTVC AGGCARCKGP LPTDCCHEQC AAGCTGPKHS DCLACLHFNH SGICELHCPA LVTYNTDTFE SMPNPEGRYT FGASCVTACP

301 YNYLSTDVGS CTLVCPLHNQ EVTAEDGTQR CEKCSKPCAR VCYGLGMEHL REVRAVTSAN IQEFAGCKKI FGSLAFLPES FDGDPASNTA PLQPEQLQVF

401 ETLEEITGYL YISAWPDSLP DLSVFQNLQV IRGRILHNGA YSLTLQGLGI SWLGLRSLRE LGSGLALIHH NTHLCFVHTV PWDQLFRNPH QALLHTANRP

501 EDECVGEGLA CHQLCARGHC WGPGPTQCVN CSQFLRGQEC VEECRVLQGL PREYVNARHC LPCHPECQPQ NGSVTCFGPE ADQCVACAHY KDPPFCVARC

601 PSGVKPDLSY MPIWKFPDEE GACQPCPINC THSCVDLDDK GCPAE (SEQ ID NO: 13)
```

FIG._1A

7C2	aa	22-53	(31 RESIDUES)
7F3	aa	22-53	(31 RESIDUES)
2C4	aa	22-584	(562 RESIDUES)
7D3	aa	22-584	(562 RESIDUES)
3E8	aa	512-625	(113 RESIDUES)
4D5	aa	529-625	(96 RESIDUES)
2H11	aa	529-645	(116 RESIDUES)
3H4	aa	541-599	(58 RESIDUES)

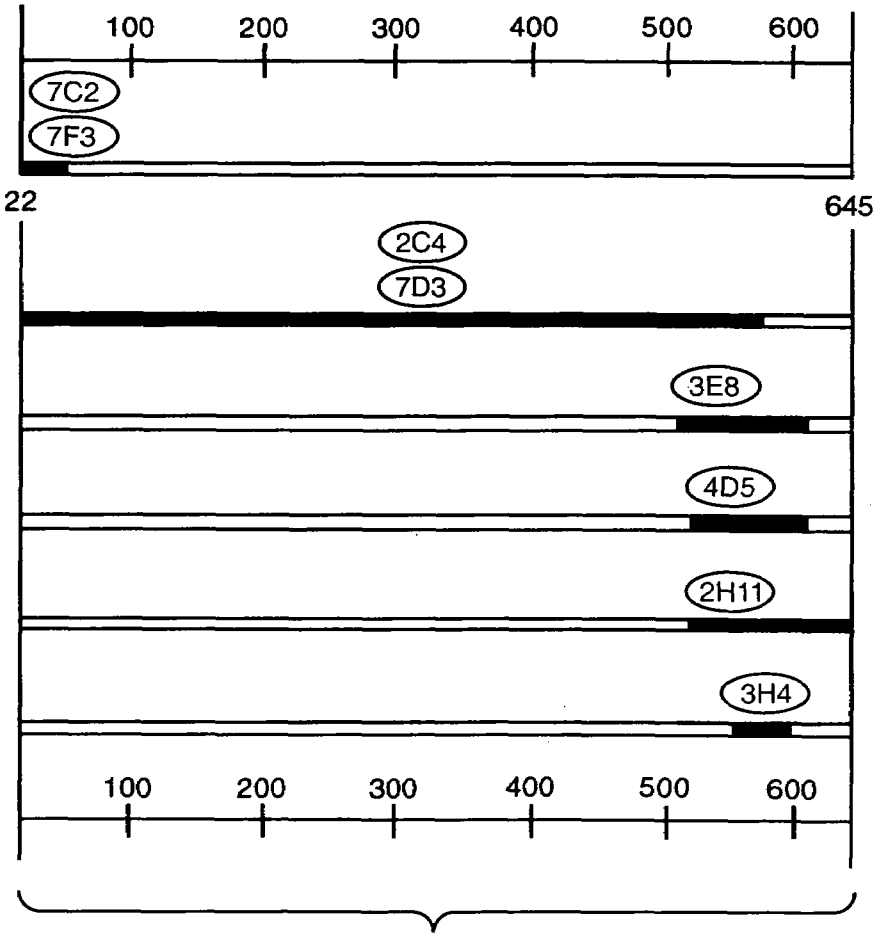


FIG._1B

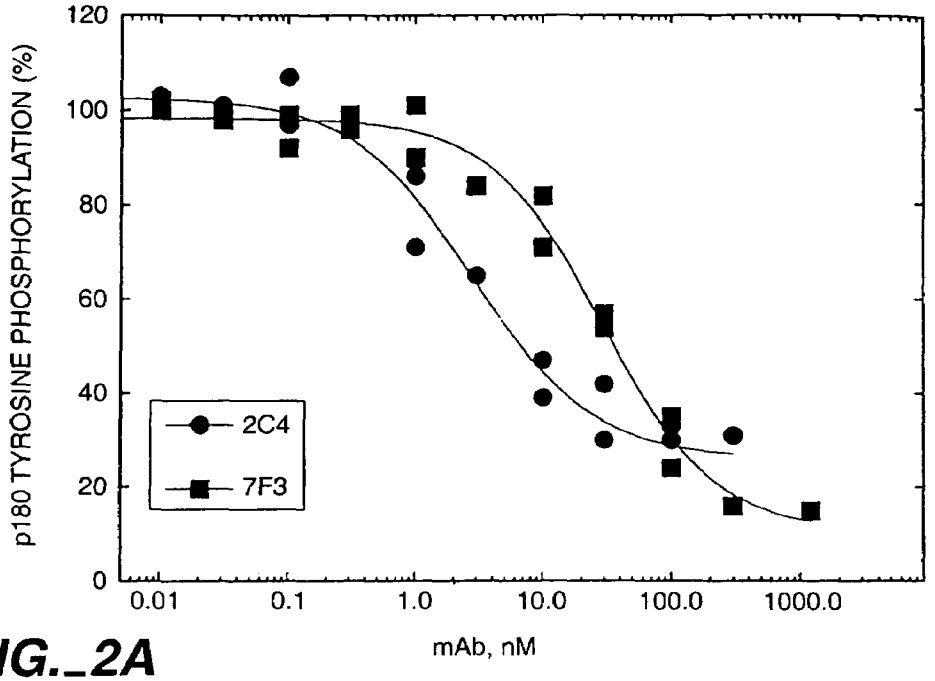

120
100
80
60
40
20
0
p180 TYROSINE PHOSPHORYLATION (%)
2C4
7F3
0.01
0.1
1.0
10.0
100.0
1000.0
mAb, nM

FIG._2A

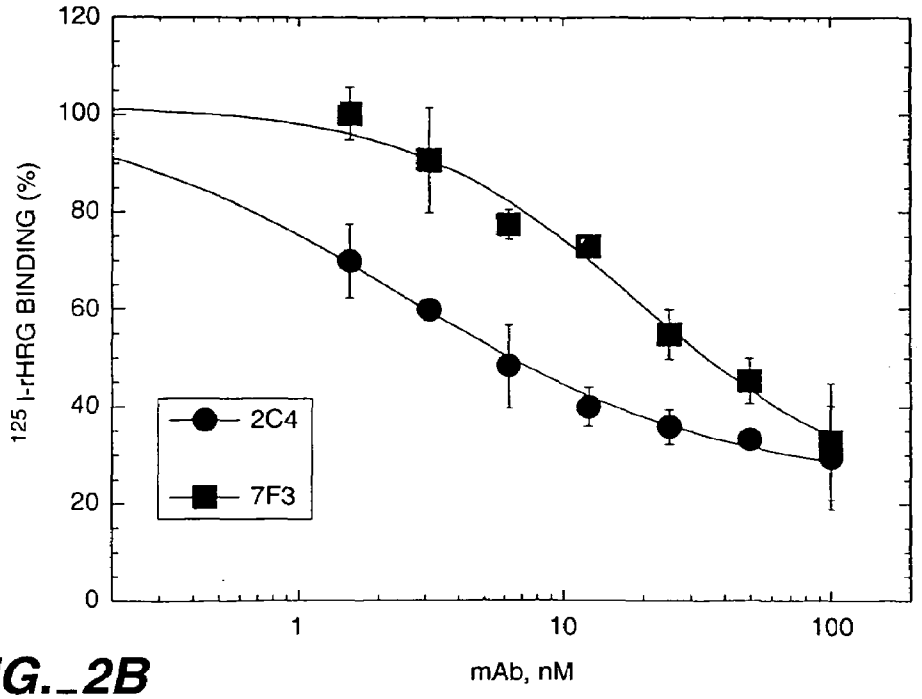

120
100
80
60
40
20
0
125 I-rHRG BINDING (%)
2C4
7F3
1
10
100
mAb, nM

FIG._2B

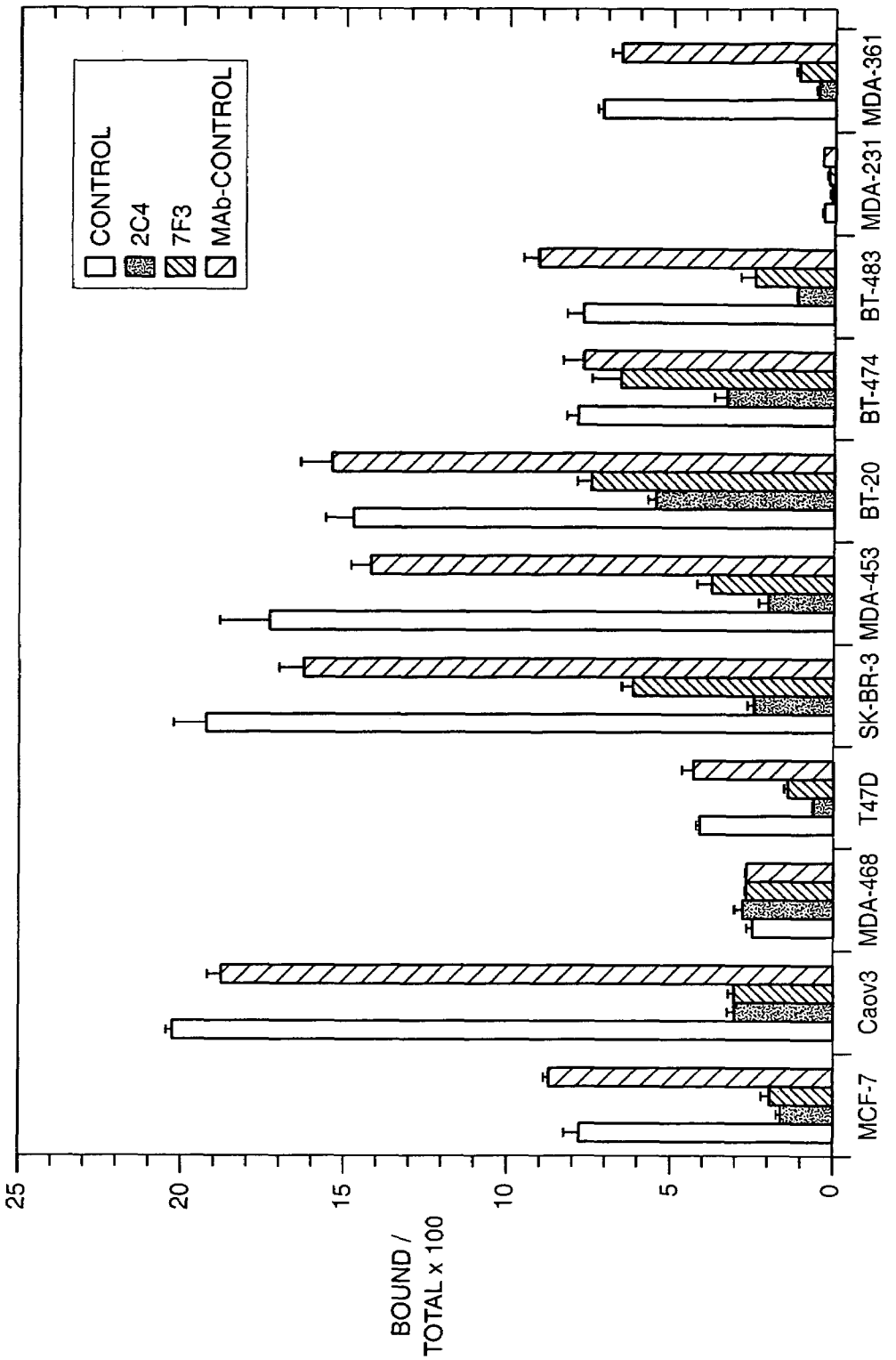

CONTROL
2C4
7F3
MAb-CONTROL
BOUND / TOTAL x 100
0
5
10
15
20
25
MCF-7
Caov3
MDA-468
T47D
SK-BR-3
MDA-453
BT-20
BT-474
BT-483
MDA-231
MDA-361

FIG._3

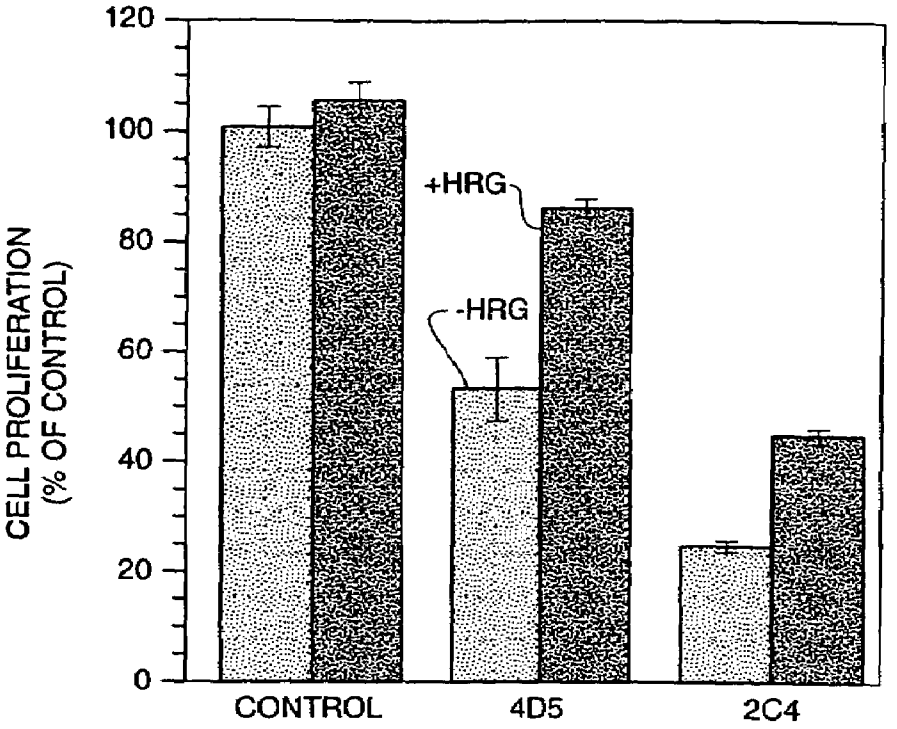

120
100
80
60
40
20
0
CELL PROLIFERATION (% OF CONTROL)
+HRG
-HRG
CONTROL
4D5
2C4

FIG._4A

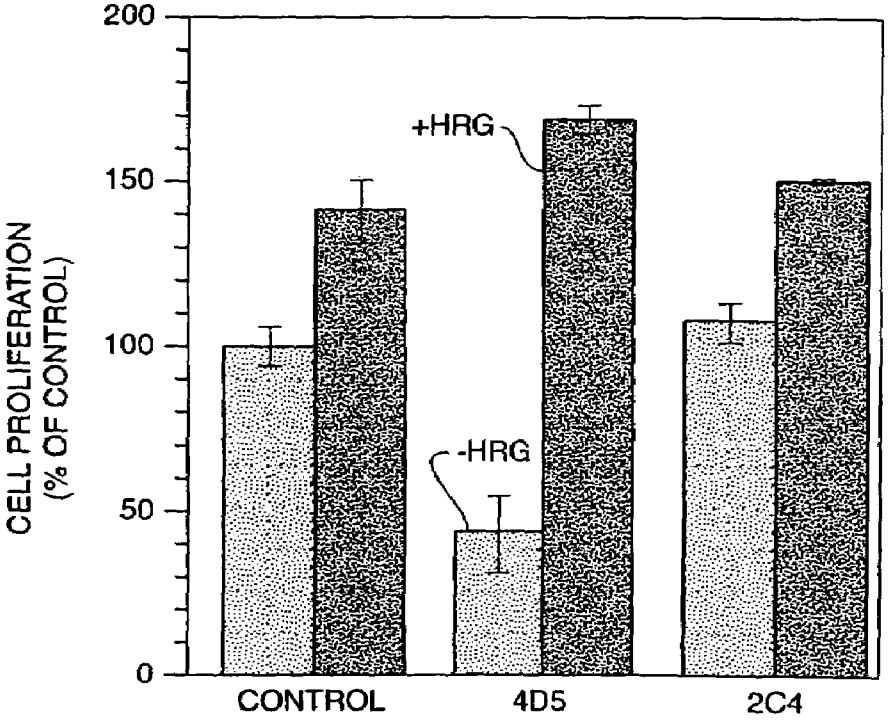
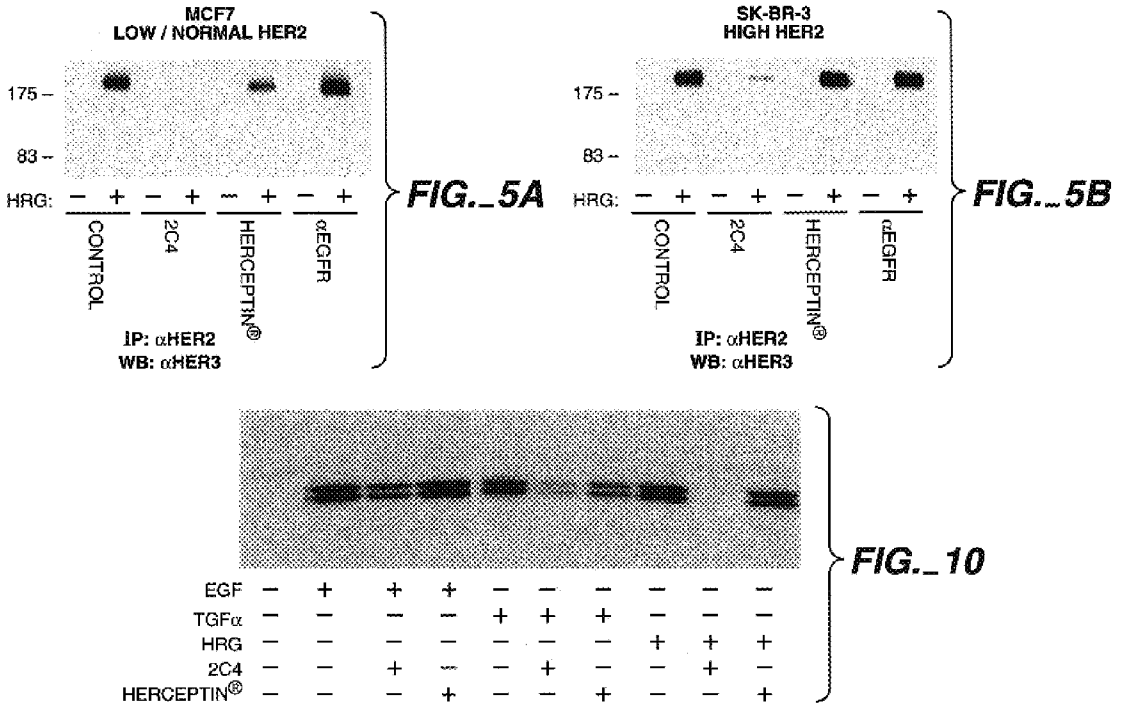

200
150
100
50
0
CELL PROLIFERATION (% OF CONTROL)
+HRG
-HRG
CONTROL
4D5
2C4

FIG._4B


MCF7
LOW / NORMAL HER2
175 –
83 –
HRG: – + – + – + – +
CONTROL
2C4
HERCEPTIN®
αEGFR
IP: αHER2
WB: αHER3
FIG._5A
SK-BR-3
HIGH HER2
175 –
83 –
HRG: – + – + – + – +
CONTROL
2C4
HERCEPTIN®
αEGFR
IP: αHER2
WB: αHER3
FIG._5B
EGF – + + + – – – – – –
TGFα – – – – + + + – – –
HRG – – – – – – – + + +
2C4 – – + – – + – – + –
HERCEPTIN® – – – + – – + – – +
FIG._10

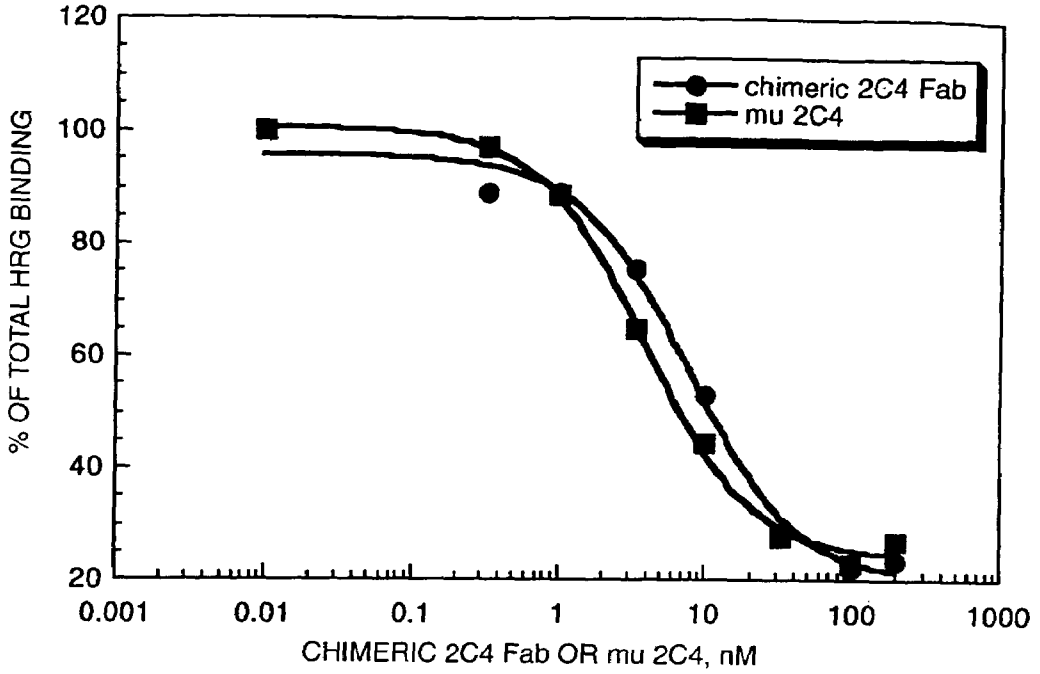

120
100
80
60
40
20
% OF TOTAL HRG BINDING
chimeric 2C4 Fab
mu 2C4
0.001
0.01
0.1
1
10
100
1000
CHIMERIC 2C4 Fab OR mu 2C4, nM

FIG._6A

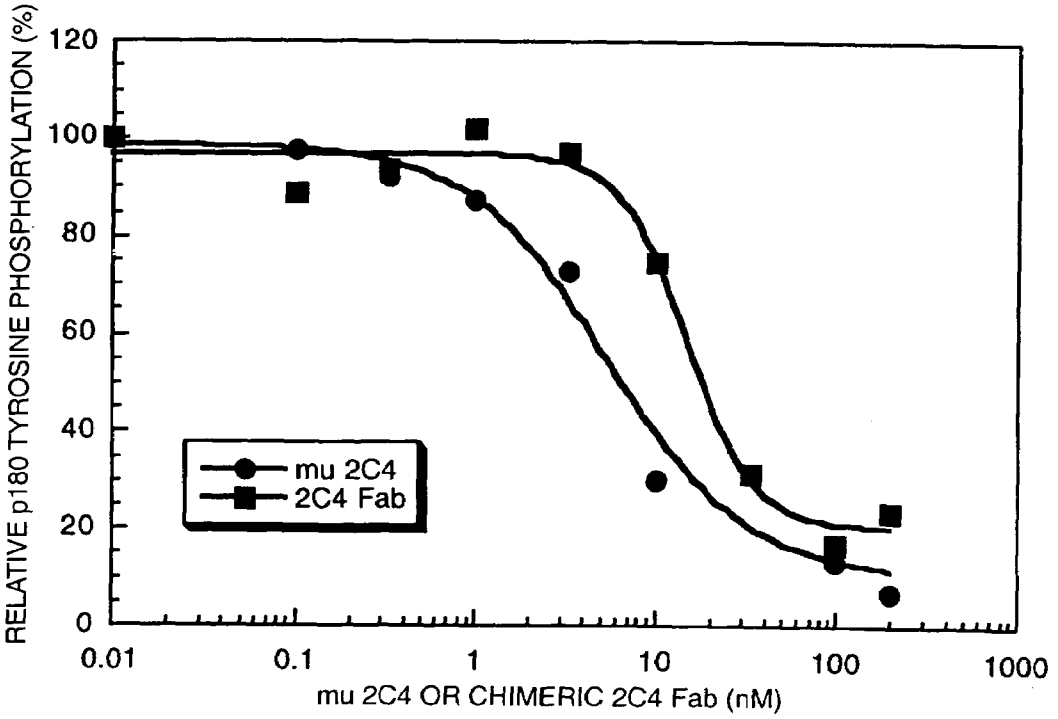

RELATIVE p180 TYROSINE PHOSPHORYLATION (%)
120
100
80
60
40
20
0
mu 2C4
2C4 Fab
0.01
0.1
1
10
100
1000
mu 2C4 OR CHIMERIC 2C4 Fab (nM)

FIG._6B

VARIABLE LIGHT

```
                10        20          30          40
2C4      DTVMTQSHKIMSTSVGDRVSITC [KASQDVSIGVA] WYQQRP
          **    **** *      *                    *
574      DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA] WYQQKP
                                  *   ** ***
hum κI   DIQMTQSPSSLSASVGDRVTITC [RASQSISNYLA] WYQQKP

                50          60         70         80
2C4      GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA
          **                  *  *         *    * *
574      GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISSLQP
                   * *****
hum κI   GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP

                90         100
2C4      EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:1)
           * *                 *   *
574      EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:3)
                     *** *
hum κI   EDFATYYC [QQYNSLPWT] FGQGTKVEIK (SEQ ID NO:5)
```

FIG._7A

VARIABLE HEAVY

```
                10        20          30          40
2C4      EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS
             **  **  *  * ***  *                  * *
574      EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA
                                    ** * *
hum III  EVQLVESGGGLVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

                50  a      60          70         80
2C4      HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM
         *  *   **                    *** *    **** *
574      PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL
                   ****** *** ****       * * *
hum III  PGKGLEWVA [VISGDGGSTYYADSVKG] RFTISRDNSKNTLYL

           abc      90         100ab        110
2C4      ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLTVSS (SEQ ID NO:2)
         ***  **                                **
574      QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTLVTVSS (SEQ ID NO:4)
                           ********
hum III  QMNSLRAEDTAVYYCAR [GRVGYSLYDY] WGQGTLVTVSS (SEQ ID NO:6)
```

FIG._7B

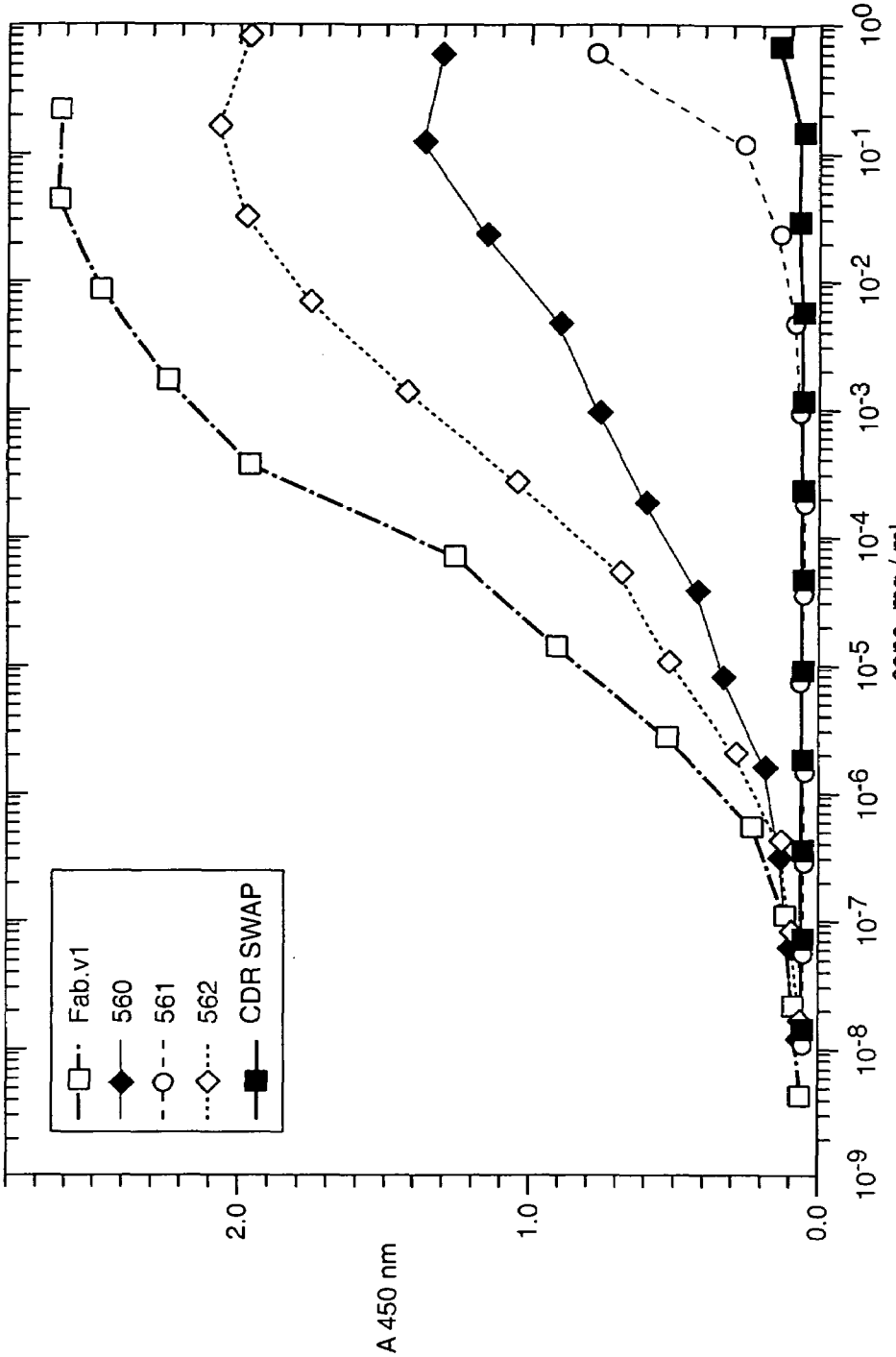
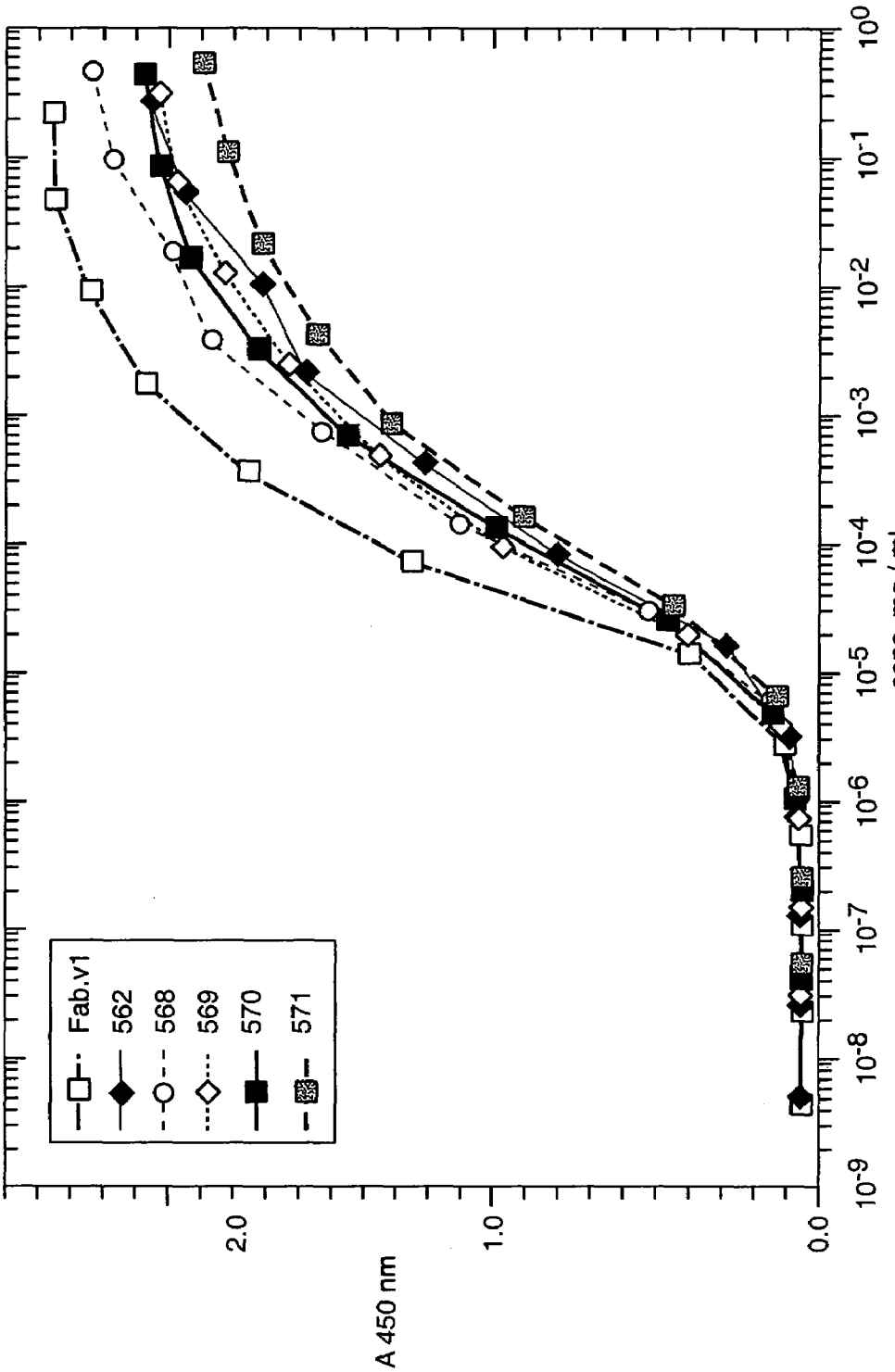

A 450 nm
2.0
1.0
0.0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
conc, mg / ml
Fab.v1
560
561
562
CDR SWAP

FIG._8A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1.0
2.0
conc, mg / ml
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10^{-1}
10^{-2}
10^{-3}
10^{-4}
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10^{-7}
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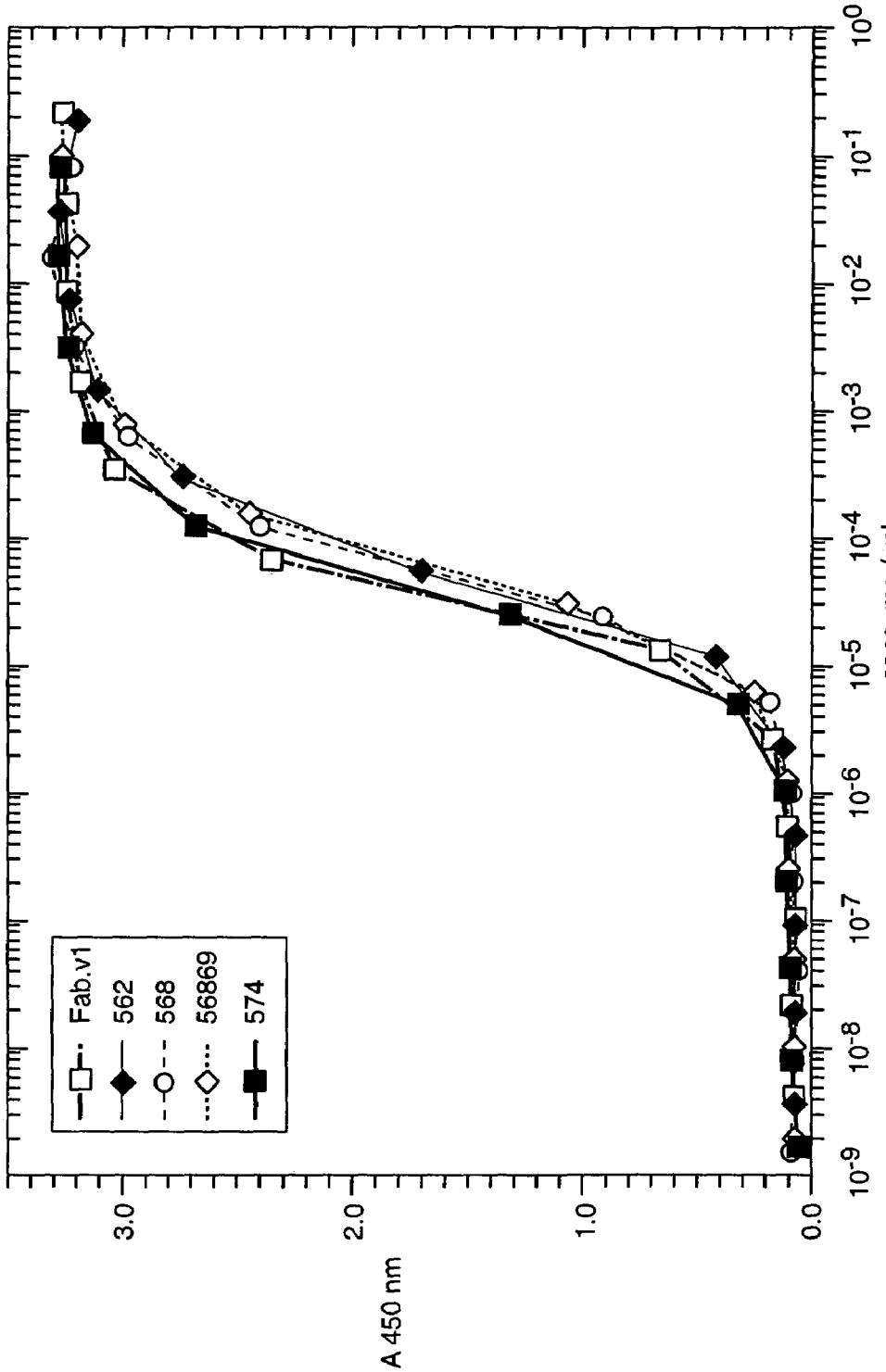
10^{-9}
Fab.v1
562
568
569
570
571

FIG._8B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562
568
56869
574
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2.0
1.0
0.0
conc, mg / ml
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10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}

FIG._8C

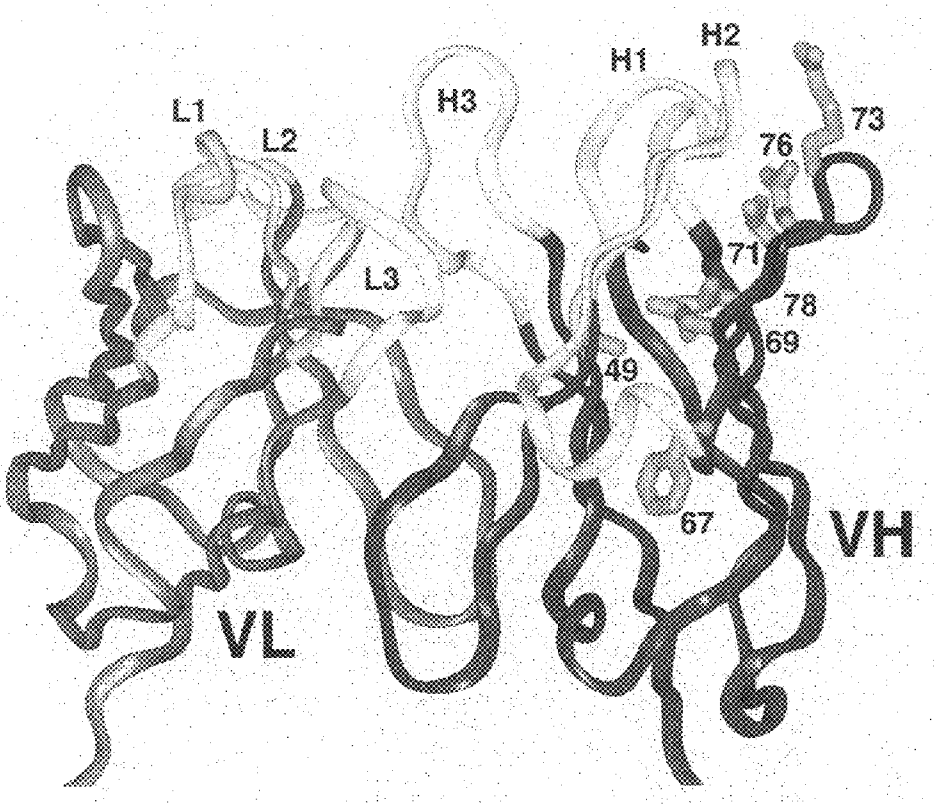

FIG._9

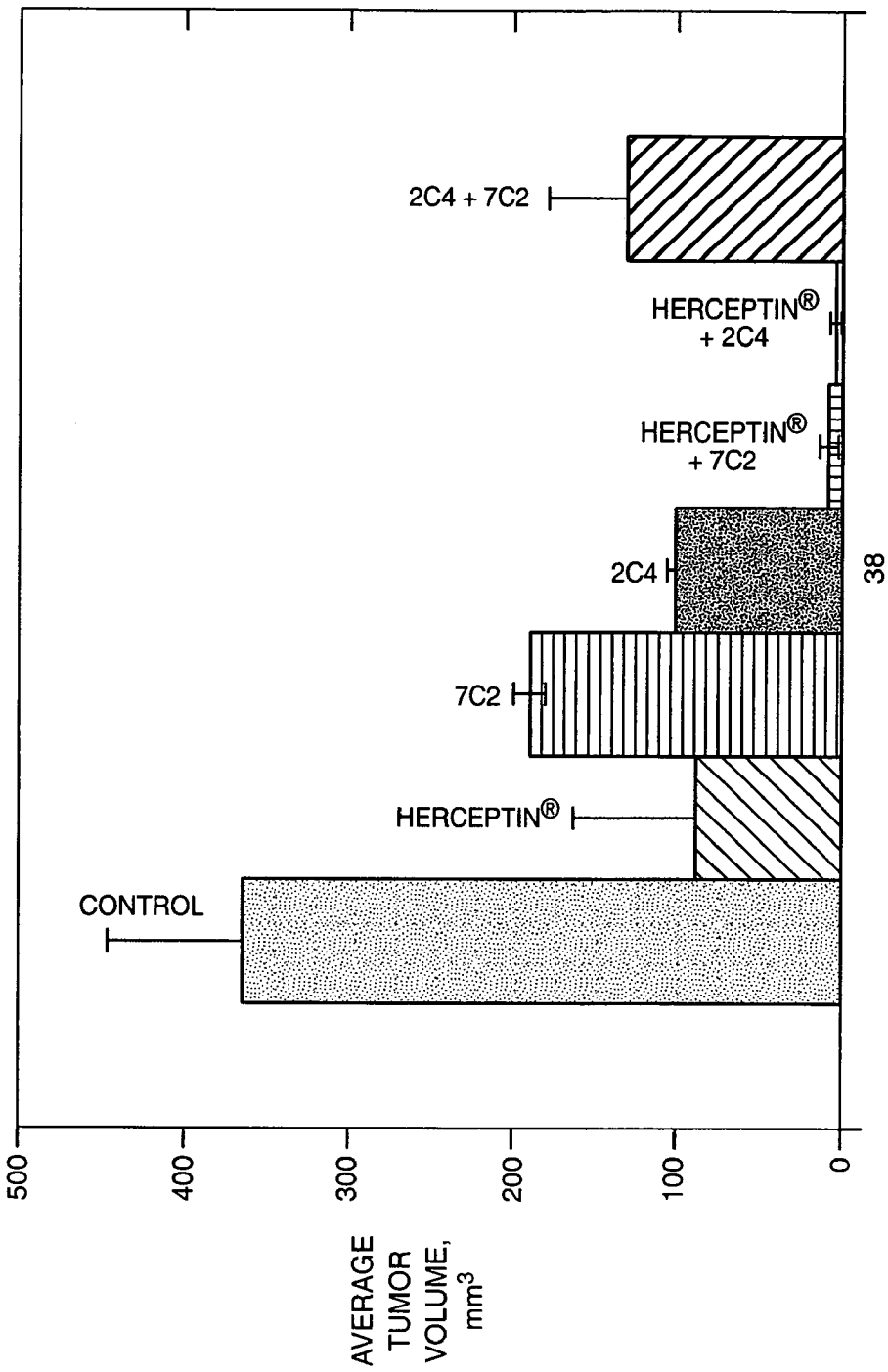
2C4 + 7C2
HERCEPTIN®
+ 2C4
HERCEPTIN®
+ 7C2
2C4
7C2
HERCEPTIN®
CONTROL
38
DAY
500
400
300
200
100
0
AVERAGE
TUMOR
VOLUME,
mm3

FIG._11

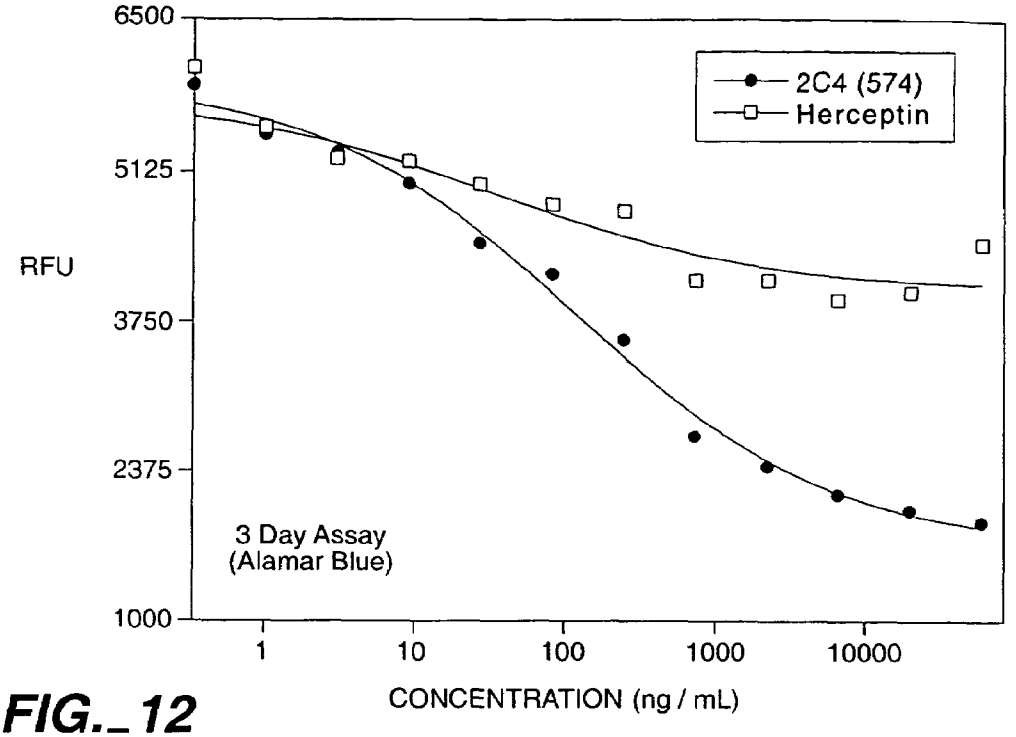
6500
5125
3750
2375
1000
RFU
2C4 (574)
Herceptin
3 Day Assay
(Alamar Blue)
1
10
100
1000
10000
CONCENTRATION (ng / mL)

FIG._12

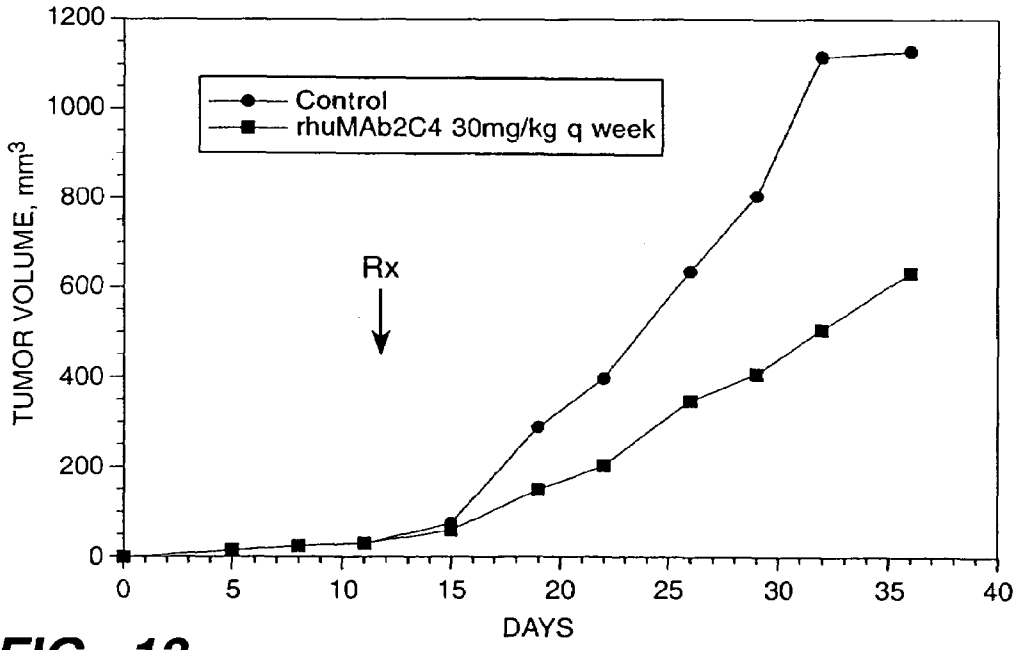
1200
1000
800
600
400
200
0
TUMOR VOLUME, mm3
Control
rhuMAb2C4 30mg/kg q week
Rx
0
5
10
15
20
25
30
35
40
DAYS

FIG._13

TREATMENT WITH ANTI-ERBB2 ANTIBODY COMBINATIONS

RELATED APPLICATIONS

This application is a divisional of U.S. Ser. No. 09/602,812 filed Jun. 23, 2000, now U.S. Pat. No. 6,949,245 which is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application No. 60/141,316 filed Jun. 25, 1999, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns humanized anti-ErbB2 antibodies and methods for treating cancer with anti-ErbB2 antibodies, such as humanized anti-ErbB2 antibodies.

BACKGROUND OF THE INVENTION

The ErbB family of receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes four distinct members including epidermal growth factor receptor (EGFR or ErbB1), HER2 (ErbB2 or p185^{neu}), HER3 (ErbB3) and HER4 (ErbB4 or tyro2).

EGFR, encoded by the erbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, transforming growth factor alpha (TGF-α), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn *Pharmac. Ther.* 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-α and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn., supra; Masui et al. *Cancer Research* 44:1002-1007 (1984); and Wu et al. *J. Clin. Invest.* 95:1897-1905 (1995).

The second member of the ErbB family, p185^{neu}, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., *Science,* 235:177-182 (1987); Slamon et al., *Science,* 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of ErbB2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. See, among others, King et al., *Science,* 229:974 (1985); Yokota et al., *Lancet:* 1:765-767 (1986); Fukushige et al., *Mol Cell Biol.,* 6:955-958 (1986); Guerin et al., *Oncogene Res.,* 3:21-31 (1988); Cohen et al., *Oncogene,* 4:81-88 (1989); Yonemura et al., *Cancer Res.,* 51:1034 (1991); Borst et al., *Gynecol. Oncol.,* 38:364 (1990); Weiner et al., *Cancer Res.,* 50:421-425 (1990); Kern et al., *Cancer Res.,* 50:5184 (1990); Park et al., *Cancer Res.,* 49:6605 (1989); Zhau et al., *Mol. Carcinog.,* 3:254-257 (1990); Aasland et al. *Br. J. Cancer* 57:358-363 (1988); Williams et al. *Pathobiology* 59:46-52 (1991); and McCann et al., *Cancer,* 65:88-92 (1990).

ErbB2 may be overexpressed in prostate cancer (Gu et al. *Cancer Lett.* 99:185-9 (1996); Ross et al. *Hum. Pathol.* 28:827-33 (1997); Ross et al. *Cancer* 79:2162-70 (1997); and Sadasivan et al. *J. Urol.* 150:126-31 (1993)).

Antibodies directed against the rat p185^{neu} and human ErbB2 protein products have been described. Drebin and colleagues have raised antibodies against the rat neu gene product, p185^{neu} See, for example, Drebin et al., *Cell* 41:695-706 (1985); Myers et al., *Meth. Enzym.* 198:277-290 (1991); and WO94/22478. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions of p185^{neu} result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. See also U.S. Pat. No. 5,824,311 issued Oct. 20, 1998.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SK-BR-3. Relative cell proliferation of the SK-BR-3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize ErbB2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also U.S. Pat. No. 5,677,171 issued Oct. 14, 1997. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis et al. *Cancer Research* 56:1457-1465 (1996); and Schaefer et al. *Oncogene* 15:1385-1394 (1997).

A recombinant humanized version of the murine anti-ErbB2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2 or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with ErbB2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). HERCEPTIN® received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the ErbB2 protein.

Other anti-ErbB2 antibodies with various properties have been described in Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991); McKenzie et al. *Oncogene* 4:543-548 (1989); Maier et al. *Cancer Res.* 51:5361-5369 (1991); Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al. *PNAS (USA)* 88:8691-8695 (1991); Bacus et al. *Cancer Research* 52:2580-2589 (1992); Xu et al. *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992); Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al. *Oncogene* 14:2099-2109 (1997).

Homology screening has resulted in the identification of two other ErbB receptor family members; ErbB3 (U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS*

(*USA*) 86:9193-9197 (1989)) and ErbB4 (EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The ErbB receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of ErbB ligands (Earp et al. *Breast Cancer Research and Treatment* 35: 115-132 (1995)). EGFR is bound by six different ligands; epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin, heparin binding epidermal growth factor (HB-EGF), betacellulin and epiregulin (Groenen et al. *Growth Factors* 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for ErbB3 and ErbB4. The heregulin family includes alpha, beta and gamma heregulins (Holmes et al., *Science,* 256:1205-1210(1992); U.S. Pat. No. 5,641,869; and Schaefer et al. *Oncogene* 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motor neuron derived factor (SMDF). For a review, see Groenen et al. *Growth Factors* 11:235-257 (1994); Lemke, G. *Molec. & Cell. Neurosci.* 7:247-262 (1996) and Lee et al. *Pharm. Rev.* 47:51-85 (1995). Recently three additional ErbB ligands were identified; neuregulin-2 (NRG-2) which is reported to bind either ErbB3 or ErbB4 (Chang et al. *Nature* 387 509-512 (1997); and Carraway et al *Nature* 387:512-516 (1997)); neuregulin-3 which binds ErbB4 (Zhang et al. *PNAS* (*USA*) 94(18):9562-7 (1997)); and neuregulin-4 which binds ErbB4 (Harari et al. *Oncogene* 18:2681-89 (1999)) HB-EGF, betacellulin and epiregulin also bind to ErbB4.

While EGF and TGFα do not bind ErbB2, EGF stimulates EGFR and ErbB2 to form a heterodimer, which activates EGFR and results in transphosphorylation of ErbB2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the ErbB2 tyrosine kinase. See Earp et al., supra. Likewise, when ErbB3 is co-expressed with ErbB2, an active signaling complex is formed and antibodies directed against ErbB2 are capable of disrupting this complex (Sliwkowski et al., *J. Biol. Chem.,* 269(20):14661-14665 (1994)). Additionally, the affinity of ErbB3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with ErbB2. See also, Levi et al., *Journal of Neuroscience* 15: 1329-1340 (1995); Morrissey et al., *Proc. Natl. Acad. Sci. USA* 92: 1431-1435 (1995); and Lewis et al., *Cancer Res.,* 56:1457-1465 (1996) with respect to the ErbB2-ErbB3 protein complex. ErbB4, like ErbB3, forms an active signaling complex with ErbB2 (Carraway and Cantley, *Cell* 78:5-8 (1994)).

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a method of treating cancer in a human, wherein the cancer expresses epidermal growth factor receptor (EGFR), comprising administering to the human a therapeutically effective amount of an antibody which binds ErbB2.

Various advantages in using an antibody which binds ErbB2 to treat such cancer, as opposed to EGFR-targeted drugs, are contemplated herein. In particular, EGFR is highly expressed in liver and skin and this provides an enormous sink for active drug where the drug binds to EGFR. In addition, skin toxicity has been observed for other EGFR-targeted drugs such as the chimeric anti-EGFR antibody C225 and the small molecule drug ZD1839 which binds EGFR. Antibodies which bind ErbB2 are anticipated to have a better safety profile than such drugs.

Where the antibody used for therapy herein blocks ligand activation of an ErbB receptor and/or has a biological characteristic of monoclonal antibody 2C4, further advantages are achieved. For example, while EGFR-targeted drugs interfere only with EGFR, the antibodies of particular interest herein (e.g. 2C4, including humanized and/or affinity matured variants thereof) will interfere with EGFR/ErbB2, ErbB3/ErbB4 and ErbB2/ErbB3 heterodimers. In addition, the antibodies herein that bind ErbB2 and block ligand activation of an ErbB receptor will be complementary to EGFR-targeted drugs, where EGFR-targeted drugs are not complementary to each other.

The invention further provides a method of treating cancer in a human, wherein the cancer is not characterized by overexpression of the ErbB2 receptor, comprising administering to the human a therapeutically effective amount of an antibody which binds to ErbB2 and blocks ligand activation of an ErbB receptor.

In addition, the present invention provides a method of treating hormone independent cancer in a human comprising administering to the human a therapeutically effective amount of an antibody which binds ErbB2 receptor, and blocks ligand activation of an ErbB receptor.

The invention further provides a method of treating cancer in a human comprising administering to the human therapeutically effective amounts of (a) a first antibody which binds ErbB2 and inhibits growth of cancer cells which overexpress ErbB2; and (b) a second antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor.

The invention also provides a method of treating a cancer in a human, wherein the cancer is selected from the group consisting of colon, rectal and colorectal cancer, comprising administering to the human a therapeutically effective amount of an antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor.

In further embodiments, the invention provides articles of manufacture for use (among other things) in the above methods. For example, the invention provides an article of manufacture comprising a container and a composition contained therein, wherein the composition comprises an antibody which binds ErbB2, and further comprising a package insert indicating that the composition can be used to treat cancer which expresses epidermal growth factor receptor (EGFR).

The invention additionally pertains to an article of manufacture comprising a container and a composition contained therein, wherein the composition comprises an antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor, and further comprising a package insert indicating that the composition can be used to treat cancer, wherein the cancer is not characterized by overexpression of the ErbB2 receptor.

Also, the invention relates to an article of manufacture comprising a container and a composition contained therein, wherein the composition comprises an antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor, and further comprising a package insert indicating that the composition can be used to treat hormone independent cancer.

In a further embodiment, an article of manufacture is provided which comprises (a) a first container with a composition contained therein, wherein the composition comprises a first antibody which binds ErbB2 and inhibits growth of cancer cells which overexpress ErbB2; and (b) a second container with a composition contained therein, wherein the

composition comprises a second antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor.

A further article of manufacture is provided which comprises a container and a composition contained therein, wherein the composition comprises an antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor, and further comprises a package insert indicating that the composition can be used to treat a cancer selected from the group consisting of colon, rectal and colorectal cancer.

The invention additionally provides: a humanized antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor; a composition comprising the humanized antibody and a pharmaceutically acceptable carrier; and an immunoconjugate comprising the humanized antibody conjugated with a cytotoxic agent.

Moreover, the invention provides isolated nucleic acid encoding the humanized antibody; a vector comprising the nucleic acid; a host cell comprising the nucleic acid or the vector; as well as a process of producing the humanized antibody comprising culturing a host cell comprising the nucleic acid so that the nucleic acid is expressed and, optionally, further comprising recovering the humanized antibody from the host cell culture (e.g. from the host cell culture medium).

The invention further pertains to an immunoconjugate comprising an antibody which binds ErbB2 conjugated to one or more calicheamicin molecules, and the use of such conjugates for treating ErbB2 expressing cancer, e.g., ErbB2 overexpressing cancer, in a human. Preferably, the antibody in the conjugate is monoclonal antibody 4D5, e.g., humanized 4D5 (and preferably huMAb4D5-8 (HERCEPTIN®); or monoclonal antibody 2C4, e.g., humanized 2C4. The antibody in the immunoconjugate may be an intact antibody (e.g., an intact IgG_1 antibody) or an antibody fragment (e.g. a Fab, $F(ab')_2$, diabody etc).

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A and **1**B depict epitope mapping of residues 22-645 within the extracellular domain (ECD) of ErbB2 (amino acid sequence, including signal sequence, shown in FIG. **1**A; SEQ ID NO:13) as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 (1993); and Renz et al. *J. Cell Biol.* 125(6): 1395-1406(1994)). The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293 cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested and either the anti-ErbB2 monoclonal antibodies or control antibodies were added to the supernatant and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. As shown in FIG. **1**B, the anti-ErbB2 antibodies 7C2, 7F3, 2C4, 7D3, 3E8, 4D5, 2H11 and 3H4 bind various ErbB2 ECD epitopes.

FIGS. **2**A and **2**B show the effect of anti-ErbB2 monoclonal antibodies 2C4 and 7F3 on rHRGβ1 activation of MCF7 cells. FIG. **2**A shows dose-response curves for 2C4 or 7F3 inhibition of HRG stimulation of tyrosine phosphorylation. FIG. **2**B shows dose-response curves for the inhibition of ^{125}I-labeled $rHRG\beta1_{177-244}$ binding to MCF7 cells by 2C4 or 7F3.

FIG. **3** depicts inhibition of specific ^{125}I-labeled $rHRG\beta1_{177-244}$ binding to a panel of human tumor cell lines by the anti-ErbB2 monoclonal antibodies 2C4 or 7F3. Monoclonal antibody-controls are isotype-matched murine monoclonal antibodies that do not block rHRG binding. Nonspecific ^{125}I-labeled $rHRG\beta1_{177-244}$ binding was determined from parallel incubations performed in the presence of 100 nM rHRGβ1. Values for nonspecific ^{125}I-labeled $rHRG\beta1_{177-244}$ binding were less than 1% of the total for all the cell lines tested.

FIGS. **4**A and **4**B show the effect of monoclonal antibodies 2C4 and 4D5 on proliferation of MDA-MB-175 (FIG. **4**A) and SK-BR-3 (FIG. **4**B) cells. MDA-MB-175 and SK-BR-3 cells were seeded in 96 well plates and allowed to adhere for 2 hours. Experiment was carried out in medium containing 1% serum. Anti-ErbB2 antibodies or medium alone were added and the cells were incubated for 2 hours at 37° C. Subsequently rHRGβ1 (1 nM) or medium alone were added and the cells were incubated for 4 days. Monolayers were washed and stained/fixed with 0.5% crystal violet. To determine cell proliferation the absorbance was measured at 540 nm.

FIGS. **5**A and **5**B show the effect of monoclonal antibody 2C4, HERCEPTIN® antibody or an anti-EGFR antibody on heregulin (HRG) dependent association of ErbB2 with ErbB3 in MCF7 cells expressing low/normal levels of ErbB2 (FIG. **5**A) and SK-BR-3 cells expressing high levels of ErbB2 (FIG. **5**B); see Example 2 below.

FIGS. **6**A and **6**B compare the activities of intact murine monoclonal antibody 2C4 (mu 2C4) and a chimeric 2C4 Fab fragment. FIG. **6**A shows inhibition of ^{125}I-HRG binding to MCF7 cells by chimeric 2C4 Fab or intact murine monoclonal antibody 2C4. MCF7 cells were seeded in 24-well plates (1×10^5 cells/well) and grown to about 85% confluency for two days. Binding experiments were conducted as described in Lewis et al. *Cancer Research* 56:1457-1465 (1996). FIG. **6**B depicts inhibition of rHRGβ1 activation of p180 tyrosine phosphorylation in MCF7 cells performed as described in Lewis et al. *Cancer Research* 56:1457-1465 (1996).

FIGS. **7**A and **7**B depict alignments of the amino acid sequences of the variable light (V_L) (FIG. **7**A) and variable heavy (V_H) (FIG. **7**B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 1 and 2, respectively); V_L and V_H domains of humanized 2C4 version 574 (SEQ ID Nos. 3 and 4, respectively), and human V_L and V_H consensus frameworks (hum κ1, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 5 and 6, respectively). Asterisks identify differences between humanized 2C4 version 574 and murine monoclonal antibody 2C4 or between humanized 2C4 version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets.

FIGS. **8**A to C show binding of chimeric Fab 2C4 (Fab.v1) and several humanized 2C4 variants to ErbB2 extracellular domain (ECD) as determined by ELISA in Example 3.

FIG. **9** is a ribbon diagram of the V_L and V_H domains of monoclonal antibody 2C4 with white CDR backbone labeled (L1, L2, L3, H1, H2, H3). V_H sidechains evaluated by mutagenesis during humanization (see Example 3, Table 2) are also shown.

FIG. **10** depicts the effect of monoclonal antibody 2C4 or HERCEPTIN® on EGF, TGF-α, or HRG-mediated activation of mitogen-activated protein kinase (MAPK).

FIG. **11** is a bar graph showing the effect of anti-ErbB2 antibodies (alone or in combinations) on Calu3 lung adenocarcinoma xenografts (3+ErbB2 overexpressor). Note: treatment was stopped on day 24.

FIG. **12** depicts the effect of recombinant humanized monoclonal antibody 2C4 (rhuMAb 2C4) or HERCEPTIN® on the growth of MDA-175 cells as assessed in an Alamar Blue assay.

FIG. **13** shows the efficacy of rhuMAb 2C4 against MCF7 xenografts.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

An "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, ErbB2, ErbB3 and ErbB4 receptors and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a "native sequence" ErbB receptor or an "amino acid sequence variant" thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881-914 (1987), including naturally occurring mutant forms thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS (USA)* 87:4207-4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product.

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to human HER2 protein described, for example, in Semba et al., *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). The term "erbB2" refers to the gene encoding human ErbB2 and "neu" refers to the gene encoding rat $p185^{neu}$. Preferred ErbB2 is native sequence human ErbB2.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS (USA)* 86:9193-9197 (1989).

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-475 (1993), including isoforms thereof, e.g., as disclosed in WO99/19488, published Apr. 22, 1999.

By "ErbB ligand" is meant a polypeptide which binds to and/or activates an ErbB receptor. The ErbB ligand of particular interest herein is a native sequence human ErbB ligand such as epidermal growth factor (EGF) (Savage et al., *J. Biol. Chem.* 247:7612-7621 (1972)); transforming growth factor alpha (TGF-α) (Marquardt et al., *Science* 223:1079-1082 (1984)); amphiregulin also known as schwanoma or keratinocyte autocrine growth factor (Shoyab et al. *Science* 243:1074-1076 (1989); Kimura et al. *Nature* 348:257-260 (1990); and Cook et al. *Mol. Cell. Biol.* 11:2547-2557 (1991)); betacellulin (Shing et al., *Science* 259:1604-1607 (1993); and Sasada et al. *Biochem. Biophys. Res. Commun.* 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., *Science* 251:936-939 (1991)); epiregulin (Toyoda et al., *J. Biol. Chem.* 270:7495-7500 (1995); and Komurasaki et al. *Oncogene* 15:2841-2848 (1997)); a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., *Nature* 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., *Proc. Natl. Acad. Sci.* 94:9562-9567 (1997)); neuregulin-4 (NRG-4) (Harari et al. *Oncogene* 18:2681-89 (1999)) or cripto (CR-1) (Kannan et al. *J. Biol. Chem.* 272(6):3330-3335 (1997)). ErbB ligands which bind EGFR include EGF, TGF-α, amphiregulin, betacellulin, HB-EGF and epiregulin. ErbB ligands which bind ErbB3 include heregulins. ErbB ligands capable of binding ErbB4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3, NRG-4 and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide encoded by the heregulin gene product as disclosed in U.S. Pat. No. 5,641,869 or Marchionni et al., *Nature,* 362:312-318 (1993). Examples of heregulins include heregulin-α, heregulin-β1, heregulin-β2 and heregulin-β3 (Holmes et al., *Science,* 256:1205-1210 (1992); and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. *Cell* 69:205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. *Cell* 72:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al., *Nature,* 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho et al. *J. Biol. Chem.* 270:14523-14532 (1995)); γ-heregulin (Schaefer et al. *Oncogene* 15:1385-1394 (1997)). The term includes biologically active fragments and/or amino acid sequence variants of a native sequence HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta1_{177-244}$)

An "ErbB hetero-oligomer" herein is a noncovalently associated oligomer comprising at least two different ErbB receptors. Such complexes may form when a cell expressing two or more ErbB receptors is exposed to an ErbB ligand and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20):14661-14665 (1994), for example. Examples of such ErbB hetero-oligomers include EGFR-ErbB2, ErbB2-ErbB3 and ErbB3-ErbB4 complexes. Moreover, the ErbB hetero-oligomer may comprise two or more ErbB2 receptors combined with a different ErbB receptor, such as ErbB3, ErbB4 or EGFR. Other proteins, such as a cytokine receptor subunit (e.g. gp130) may be included in the hetero-oligomer.

By "ligand activation of an ErbB receptor" is meant signal transduction (e.g. that caused by an intracellular kinase domain of an ErbB receptor phosphorylating tyrosine residues in the ErbB receptor or a substrate polypeptide) mediated by ErbB ligand binding to a ErbB hetero-oligomer comprising the ErbB receptor of interest. Generally, this will involve binding of an ErbB ligand to an ErbB hetero-oligomer which activates a kinase domain of one or more of the ErbB receptors in the hetero-oligomer and thereby results in phosphorylation of tyrosine residues in one or more of the ErbB receptors and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s). ErbB receptor activation can be quantified using various tyrosine phosphorylation assays.

A "native sequence" polypeptide is one which has the same amino acid sequence as a polypeptide (e.g., ErbB receptor or ErbB ligand) derived from nature. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

The term "amino acid sequence variant" refers to polypeptides having amino acid sequences that differ to some extent from a native sequence polypeptide. Ordinarily, amino acid sequence variants will possess at least about 70% homology with at least one receptor binding domain of a native ErbB

ligand or with at least one ligand binding domain of a native ErbB receptor, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with such receptor or ligand binding domains. The amino acid sequence variants possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of the native amino acid sequence.

"Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. One such computer program is "Align 2", authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, D.C. 20559, on Dec. 10, 1991.

The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments, so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, Ape etc) and human constant region sequences.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragment(s).

An "intact" antibody is one which comprises an antigen-binding variable region as well as a light chain constant domain (C_L) and heavy chain constant domains, C_H1, C_H2 and C_H3. The constant domains may be native sequence constant domains (e.g. human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc.

Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes". There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS* (*USA*) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immu-

noreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)).

"Complement dependent cytotoxicity" or "CDC" refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (e.g. an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994). Anti-ErbB2 antibody scFv fragments are described in WO93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a variable heavy domain (V_H) connected to a variable light domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

"Humanized" forms of non-human (e.g., rodent) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipi-

ent antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

Humanized anti-ErbB2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 (HERCEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337 expressly incorporated herein by reference; humanized 520C9 (WO93/21319) and humanized 2C4 antibodies as described hereinbelow.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

An antibody "which binds" an antigen of interest, e.g. ErbB2 antigen, is one capable of binding that antigen with sufficient affinity such that the antibody is useful as a therapeutic agent in targeting a cell expressing the antigen. Where the antibody is one which binds ErbB2, it will usually preferentially bind ErbB2 as opposed to other ErbB receptors, and may be one which does not significantly cross-react with other proteins such as EGFR, ErbB3 or ErbB4. In such embodiments, the extent of binding of the antibody to these non-ErbB2 proteins (e.g., cell surface binding to endogenous receptor) will be less than 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA). Sometimes, the anti-ErbB2 antibody will not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984).

An antibody which "blocks" ligand activation of an ErbB receptor is one which reduces or prevents such activation as hereinabove defined, wherein the antibody is able to block ligand activation of the ErbB receptor substantially more effectively than monoclonal antibody 4D5, e.g. about as effectively as monoclonal antibodies 7F3 or 2C4 or Fab fragments thereof and preferably about as effectively as monoclonal antibody 2C4 or a Fab fragment thereof. For example, the antibody that blocks ligand activation of an ErbB receptor may be one which is about 50-100% more effective than 4D5 at blocking formation of an ErbB hetero-oligomer. Blocking of ligand activation of an ErbB receptor can occur by any means, e.g. by interfering with: ligand binding to an ErbB receptor, ErbB complex formation, tyrosine kinase activity of an ErbB receptor in an ErbB complex and/or phosphorylation of tyrosine kinase residue(s) in or by an ErbB receptor. Examples of antibodies which block ligand activation of an ErbB receptor include monoclonal antibodies 2C4 and 7F3 (which block HRG activation of ErbB2/ErbB3 and ErbB2/ErbB4 hetero-oligomers; and EGF, TGF-α, amphiregulin, HB-EGF and/or epiregulin activation of an EGFR/ErbB2 hetero-oligomer); and L26, L96 and L288 antibodies (Klapper et al. *Oncogene* 14:2099-2109 (1997)), which block EGF and NDF binding to T47D cells which express EGFR, ErbB2, ErbB3 and ErbB4.

An antibody having a "biological characteristic" of a designated antibody, such as the monoclonal antibody designated 2C4, is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen (e.g. ErbB2). For example, an antibody with a biological characteristic of 2C4 may block HRG activation of an ErbB hetero-oligomer comprising ErbB2 and ErbB3 or ErbB4; block EGF, TGF-α, HB-EGF, epiregulin and/or amphiregulin activation of an ErbB receptor comprising EGFR and ErbB2; block EGF, TGF-αand/or HRG mediated activation of MAPK; and/or bind the same epitope in the extracellular domain of ErbB2 as that bound by 2C4 (e.g. which blocks binding of monoclonal antibody 2C4 to ErbB2).

Unless indicated otherwise, the expression "monoclonal antibody 2C4" refers to an antibody that has antigen binding residues of, or derived from, the murine 2C4 antibody of the Examples below. For example, the monoclonal antibody 2C4 may be murine monoclonal antibody 2C4 or a variant thereof, such as humanized antibody 2C4, possessing antigen binding amino acid residues of murine monoclonal antibody 2C4. Examples of humanized 2C4 antibodies are provided in Example 3 below. Unless indicated otherwise, the expression "rhuMAb 2C4" when used herein refers to an antibody comprising the variable light (V_L) and variable heavy (V_H) sequences of SEQ ID Nos. 3 and 4, respectively, fused to human light and heavy IgG1 (non-A allotype) constant region sequences optionally expressed by a Chinese Hamster Ovary (CHO) cell.

Unless indicated otherwise, the term "monoclonal antibody 4D5" refers to an antibody that has antigen binding residues of, or derived from, the murine 4D5 antibody (ATCC CRL 10463). For example, the monoclonal antibody 4D5 may be murine monoclonal antibody 4D5 or a variant thereof, such as a humanized 4D5, possessing antigen binding residues of murine monoclonal antibody 4D5. Exemplary humanized 4D5 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 (HERCEPTIN®) as in U.S. Pat. No. 5,821,337, with huMAb4D5-8 (HERCEPTIN®) being a preferred humanized 4D5 antibody.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB expressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of ErbB expressing cells in S phase. Examples of growth inhibitory agents include

agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (W B Saunders: Philadelphia, 1995), especially p. 13.

Examples of "growth inhibitory" antibodies are those which bind to ErbB2 and inhibit the growth of cancer cells overexpressing ErbB2. Preferred growth inhibitory anti-ErbB2 antibodies inhibit growth of SK-BR-3 breast tumor cells in cell culture by greater than 20%, and preferably greater than 50% (e.g. from about 50% to about 100%) at an antibody concentration of about 0.5 to 30 μg/ml, where the growth inhibition is determined six days after exposure of the SK-BR-3 cells to the antibody (see U.S. Pat. No. 5,677,171 issued Oct. 14, 1997). The SK-BR-3 cell growth inhibition assay is described in more detail in that patent and hereinbelow. The preferred growth inhibitory antibody is monoclonal antibody 4D5, e.g., humanized 4D5.

An antibody which "induces cell death" is one which causes a viable cell to become nonviable. The cell is generally one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SK-BR-3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 (1995)) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the PI uptake assay in BT474 cells (see below).

An antibody which "induces apoptosis" is one which induces programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which overexpresses the ErbB2 receptor. Preferably the cell is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SK-BR-3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an annexin binding assay using BT474 cells (see below). Sometimes the pro-apoptotic antibody will be one which further blocks ErbB ligand activation of an ErbB receptor (e.g. 7F3 antibody); i.e. the antibody shares a biological characteristic with monoclonal antibody 2C4. In other situations, the antibody is one which does not significantly block ErbB ligand activation of an ErbB receptor (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The "epitope 2C4" is the region in the extracellular domain of ErbB2 to which the antibody 2C4 binds. In order to screen for antibodies which bind to the 2C4 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 2C4 epitope of ErbB2 (e.g. any one or more residues in the region from about residue 22 to about residue 584 of ErbB2, inclusive; see FIGS. **1**A-B).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane domain of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of ErbB2 (e.g. any one or more residues in the region from about residue 529 to about residue 625, inclusive; see FIGS. **1**A-B).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain; see FIGS. **1**A-B.

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (e.g. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; see FIGS. **1**A-B).

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. Hence, the mammal to be treated herein may have been diagnosed as having the disorder or may be predisposed or susceptible to the disorder.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial,

stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP) and/or determining the response rate (RR).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

An "ErbB-expressing cancer" is one comprising cells which have ErbB protein present at their cell surface. An "ErbB2-expressing cancer" is one which produces sufficient levels of ErbB2 at the surface of cells thereof, such that an anti-ErbB2 antibody can bind thereto and have a therapeutic effect with respect to the cancer.

A cancer "characterized by excessive activation" of an ErbB receptor is one in which the extent of ErbB receptor activation in cancer cells significantly exceeds the level of activation of that receptor in non-cancerous cells of the same tissue type. Such excessive activation may result from overexpression of the ErbB receptor and/or greater than normal levels of an ErbB ligand available for activating the ErbB receptor in the cancer cells. Such excessive activation may cause and/or be caused by the malignant state of a cancer cell. In some embodiments, the cancer will be subjected to a diagnostic or prognostic assay to determine whether amplification and/or overexpression of an ErbB receptor is occurring which results in such excessive activation of the ErbB receptor. Alternatively, or additionally, the cancer may be subjected to a diagnostic or prognostic assay to determine whether amplification and/or overexpression an ErbB ligand is occurring in the cancer which attributes to excessive activation of the receptor. In a subset of such cancers, excessive activation of the receptor may result from an autocrine stimulatory pathway.

In an "autocrine" stimulatory pathway, self stimulation occurs by virtue of the cancer cell producing both an ErbB ligand and its cognate ErbB receptor. For example, the cancer may express or overexpress EGFR and also express or overexpress an EGFR ligand (e.g. EGF, TGF-α, or HB-EGF). In another embodiment, the cancer may express or overexpress ErbB2 and also express or overexpress a heregulin (e.g. γ-HRG).

A cancer which "overexpresses" an ErbB receptor is one which has significantly higher levels of an ErbB receptor, such as ErbB2, at the cell surface thereof, compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. ErbB receptor overexpression may be determined in a diagnostic or prognostic assay by evaluating increased levels of the ErbB protein present on the surface of a cell (e.g. via an immunohistochemistry assay; IHC). Alternatively, or additionally, one may measure levels of ErbB-encoding nucleic acid in the cell, e.g. via fluorescent in situ hybridization (FISH; see WO98/45479 published October, 1998), southern blotting, or polymerase chain reaction (PCR) techniques, such as real time quantitative PCR (RT-PCR). One may also study ErbB receptor overexpression by measuring shed antigen (e.g., ErbB extracellular domain) in a biological fluid such as serum (see, e.g., U.S. Pat. No. 4,933,294 issued Jun. 12, 1990; WO91/05264 published Apr. 18, 1991; U.S. Pat. No. 5,401,638 issued Mar. 28, 1995; and Sias et al. *J. Immunol. Methods* 132: 73-80 (1990)). Aside from the above assays, various in vivo assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, e.g. a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, e.g. by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

Conversely, a cancer which is "not characterized by overexpression of the ErbB2 receptor" is one which, in a diagnostic assay, does not express higher than normal levels of ErbB2 receptor compared to a noncancerous cell of the same tissue type.

A cancer which "overexpresses" an ErbB ligand is one which produces significantly higher levels of that ligand compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. Overexpression of the ErbB ligand may be determined diagnostically by evaluating levels of the ligand (or nucleic acid encoding it) in the patient, e.g. in a tumor biopsy or by various diagnostic assays such as the IHC, FISH, southern blotting, PCR or in vivo assays described above.

A "hormone independent" cancer is one in which proliferation thereof is not dependent on the presence of a hormone which binds to a receptor expressed by cells in the cancer. Such cancers do not undergo clinical regression upon administration of pharmacological or surgical strategies that reduce the hormone concentration in or near the tumor. Examples of hormone independent cancers include androgen independent prostate cancer, estrogen independent breast cancer, endometrial cancer and ovarian cancer. Such cancers may begin as hormone dependent tumors and progress from a hormone-sensitive stage to a hormone-refractory tumor following antihormonal therapy.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

As used herein, the term "EGFR-targeted drug" refers to a therapeutic agent that binds to EGFR and, optionally, inhibits EGFR activation. Examples of such agents include antibodies and small molecules that bind to EGFR. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.); antibodies that bind type II mutant EGFR (U.S. Pat. No. 5,212,290); humanized and chimeric antibodies that bind EGFR as described in U.S. Pat. No. 5,891,996; and human antibodies that bind EGFR (see WO98/50433, Abgenix). The anti-EGFR antibody may be conjugated with a cyotoxic agent, thus generating an immunoconjugate (see, e.g., EP659, 439A2, Merck Patent GmbH). Examples of small molecules that bind to EGFR include ZD1839 (Astra Zeneca), CP-358774 (OSI/Pfizer) and AG1478.

An "anti-angiogenic agent" refers to a compound which blocks, or interferes with to some degree, the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or antibody that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. The preferred anti-angiogenic factor herein is an antibody that binds to Vascular Endothelial Growth Factor (VEGF).

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phe-

noxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

A "cardioprotectant" is a compound or composition which prevents or reduces myocardial dysfunction (i.e. cardiomyopathy and/or congestive heart failure) associated with administration of a drug, such as an anthracycline antibiotic and/or an anti-ErbB2 antibody, to a patient. The cardioprotectant may, for example, block or reduce a free-radical-mediated cardiotoxic effect and/or prevent or reduce oxidative-stress injury. Examples of cardioprotectants encompassed by the present definition include the iron-chelating agent dexrazoxane (ICRF-187) (Seifert et al. *The Annals of Pharmacotherapy* 28:1063-1072 (1994)); a lipid-lowering agent and/or anti-oxidant such as probucol (Singal et al. *J. Mol. Cell Cardiol.* 27:1055-1063 (1995)); amifostine (aminothiol 2-[(3-aminopropyl)amino]ethanethiol-dihydrogen phosphate ester, also called WR-2721, and the dephosphorylated cellular uptake form thereof called WR-1065) and S-3-(3-methylaminopropylamino)propylphosphorothioic acid (WR-151327), see Green et al. *Cancer Research* 54:738-741 (1994); digoxin (Bristow, M. R. In: Bristow M R, ed. *Drug-Induced Heart Disease*. New York: Elsevier 191-215 (1980)); beta-blockers such as metoprolol (Hjalmarson et al. *Drugs* 47:Suppl 4:31-9 (1994); and Shaddy et al. *Am. Heart J.* 129:197-9 (1995)); vitamin E; ascorbic acid (vitamin C); free radical scavengers such as oleanolic acid, ursolic acid and N-acetylcysteine (NAC); spin trapping compounds such as alpha-phenyl-tert-butyl nitrone (PBN); (Paracchini et al., *Anticancer Res.* 13:1607-1612 (1993)); selenoorganic compounds such as P251 (Elbesen); and the like.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the antibody nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

II. Production of Anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed, to overexpress ErbB2; or a carcinoma cell line such as SK-BR-3 cells, see Stancovski et al. *PNAS* (*USA*) 88:8691-8695 (1991)) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recom-

binant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove, described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); and Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130:151-188 (1992).

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy chain and light chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; and Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized Antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein

substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

Example 3 below describes production of exemplary humanized anti-ErbB2 antibodies which bind ErbB2 and block ligand activation of an ErbB receptor. The humanized antibody of particular interest herein blocks EGF, TGF-α and/or HRG mediated activation of MAPK essentially as effectively as murine monoclonal antibody 2C4 (or a Fab fragment thereof) and/or binds ErbB2 essentially as effectively as murine monoclonal antibody 2C4 (or a Fab fragment thereof). The humanized antibody herein may, for example, comprise nonhuman hypervariable region residues incorporated into a human variable heavy domain and may further comprise a framework region (FR) substitution at a position selected from the group consisting of 69H, 71H and 73H utilizing the variable domain numbering system set forth in Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). In one embodiment, the humanized antibody comprises FR substitutions at two or all of positions 69H, 71H and 73H.

An exemplary humanized antibody of interest herein comprises variable heavy domain complementarity determining residues GFTFTDYTMX, where X is preferrably D or S (SEQ ID NO:7); DVNPNSGGSIYNQRFKG (SEQ ID NO:8); and/or NLGPSFYFDY (SEQ ID NO:9), optionally comprising amino acid modifications of those CDR residues, e.g. where the modifications essentially maintain or improve affinity of the antibody. For example, the antibody variant of interest may have from about one to about seven or about five amino acid substitutions in the above variable heavy CDR sequences. Such antibody variants may be prepared by affinity maturation, e.g., as described below. The most preferred humanized antibody comprises the variable heavy domain amino acid sequence in SEQ ID NO:4.

The humanized antibody may comprise variable light domain complementarity determining residues KASQDVSIGVA (SEQ ID NO:10); $SASYX^1X^2X^3$, where X^1 is preferably R or L, X^2 is preferably Y or E, and X^3 is preferably T or S (SEQ ID NO:11); and/or QQYYIYPYT (SEQ ID NO:12), e.g. in addition to those variable heavy domain CDR residues in the preceding paragraph. Such humanized antibodies optionally comprise amino acid modifications of the above CDR residues, e.g. where the modifications essentially maintain or improve affinity of the antibody. For example, the antibody variant of interest may have from about one to about seven or about five amino acid substitutions in the above variable light CDR sequences. Such antibody variants may be prepared by affinity maturation, e.g., as described below. The most preferred humanized antibody comprises the variable light domain amino acid sequence in SEQ ID NO:3.

The present application also contemplates affinity matured antibodies which bind ErbB2 and block ligand activation of an ErbB receptor. The parent antibody may be a human antibody or a humanized antibody, e.g., one comprising the variable light and/or heavy sequences of SEQ ID Nos. 3 and 4, respectively (i.e. variant 574). The affinity matured antibody preferably binds to ErbB2 receptor with an affinity superior to that of murine 2C4 or variant 574 (e.g. from about two or about four fold, to about 100 fold or about 1000 fold improved affinity, e.g. as assessed using a ErbB2-extracellular domain (ECD) ELISA). Exemplary variable heavy CDR residues for substitution include H28, H30, H34, H35, H64, H96, H99, or combinations of two or more (e.g. two, three, four, five, six, or seven of these residues). Examples of variable light CDR residues for alteration include L28, L50, L53, L56, L91, L92, L93, L94, L96, L97 or combinations of two or more (e.g. two to three, four, five or up to about ten of these residues).

Various forms of the humanized antibody or affinity matured antibody are contemplated. For example, the humanized antibody or affinity matured antibody may be an antibody fragment, such as a Fab, which is optionally conjugated with one or more cytotoxic agent(s) in order to generate an immunoconjugate. Alternatively, the humanized antibody or affinity matured antibody may be an intact antibody, such as an intact IgG1 antibody.

(iv) Human Antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and U.S. Pat. Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty et al., *Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571(1993). Several sources of V-gene segments can be used for phage display. Clackson et al., *Nature,* 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., *J. Mol. Biol.* 222:581-597 (1991), or Griffith et al., *EMBO J.* 12:725-734 (1993). See, also, U.S. Pat. Nos. 5,565,332 and 5,573,905.

As discussed above, human antibodies may also be generated by in vitro activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

Human anti-ErbB2 antibodies are described in U.S. Pat. No. 5,772,997 issued Jun. 30, 1998 and WO 97/00271 published Jan. 3, 1997.

(v) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

WO 96/16673 describes a bispecific anti-ErbB2/anti-FcγRIII antibody and U.S. Pat. No. 5,837,234 discloses a bispecific anti-ErbB2/anti-FcγRI antibody. A bispecific anti-ErbB2/Fcα antibody is shown in WO98/02463. U.S. Pat. No. 5,821,337 teaches a bispecific anti-ErbB2/anti-CD3 antibody.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in U.S. Pat. No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method,

one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites.

Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

(vii) Other Amino Acid Sequence Modifications

Amino acid sequence modification(s) of the anti-ErbB2 antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the anti-ErbB2 antibody are prepared by introducing appropriate nucleotide changes into the anti-ErbB2 antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-ErbB2 antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the anti-ErbB2 antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the anti-ErbB2 antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells *Science,* 244: 1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with ErbB2 antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed anti-ErbB2 antibody variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-ErbB2 antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the anti-ErbB2 antibody molecule include the fusion to the N- or C-terminus of the anti-ErbB2 antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-ErbB2 antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the anti-ErbB2 antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human ErbB2. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of the anti-ErbB2 antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-ErbB2 antibody.

It may be desirable to modify the antibody of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced antitumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Pat. No. 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the

Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

(viii) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. One may further select antibodies with certain biological characteristics, as desired.

To identify an antibody which blocks ligand activation of an ErbB receptor, the ability of the antibody to block ErbB ligand binding to cells expressing the ErbB receptor (e.g. in conjugation with another ErbB receptor with which the ErbB receptor of interest forms an ErbB hetero-oligomer) may be determined. For example, cells naturally expressing, or transfected to express, ErbB receptors of the ErbB hetero-oligomer may be incubated with the antibody and then exposed to labeled ErbB ligand. The ability of the anti-ErbB2 antibody to block ligand binding to the ErbB receptor in the ErbB hetero-oligomer may then be evaluated.

For example, inhibition of HRG binding to MCF7 breast tumor cell lines by anti-ErbB2 antibodies may be performed using monolayer MCF7 cultures on ice in a 24-well-plate format essentially as described in Example 1 below. Anti-ErbB2 monoclonal antibodies may be added to each well and incubated for 30 minutes. ^{125}I-labeled $rHRG\beta1_{177-224}$ (25 pm) may then be added, and the incubation may be continued for 4 to 16 hours. Dose response curves may be prepared and an IC_{50} value may be calculated for the antibody of interest. In one embodiment, the antibody which blocks ligand activation of an ErbB receptor will have an IC_{50} for inhibiting HRG binding to MCF7 cells in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG binding to MCF7 cells in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

Alternatively, or additionally, the ability of the anti-ErbB2 antibody to block ErbB ligand-stimulated tyrosine phosphorylation of an ErbB receptor present in an ErbB hetero-oligomer may be assessed. For example, cells endogenously expressing the ErbB receptors or transfected to expressed them may be incubated with the antibody and then assayed for ErbB ligand-dependent tyrosine phosphorylation activity using an anti-phosphotyrosine monoclonal (which is optionally conjugated with a detectable label). The kinase receptor activation assay described in U.S. Pat. No. 5,766,863 is also available for determining ErbB receptor activation and blocking of that activity by an antibody.

In one embodiment, one may screen for an antibody which inhibits HRG stimulation of p180 tyrosine phosphorylation in MCF7 cells essentially as described in Example 1 below. For example, the MCF7 cells may be plated in 24-well plates and monoclonal antibodies to ErbB2 may be added to each well and incubated for 30 minutes at room temperature; then $rHRG\beta1_{177-244}$ may be added to each well to a final concentration of 0.2 nM, and the incubation may be continued for 8 minutes. Media may be aspirated from each well, and reactions may be stopped by the addition of 100 μl of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 μl) may be electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (at 1 μg/ml) immunoblots may be developed, and the intensity of the predominant reactive band at M_r ~180,000 may be quantified by reflectance densitometry. The antibody selected will preferably significantly inhibit HRG stimulation of p180 tyrosine phosphorylation to about 0-35% of control in this assay. A dose-response curve for inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry may be prepared and an IC_{50} for the antibody of interest may be calculated. In one embodiment, the antibody which blocks ligand activation of an ErbB receptor will have an IC_{50} for inhibiting HRG stimulation of p180 tyrosine phosphorylation in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG stimulation of p180 tyrosine phosphorylation in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

One may also assess the growth inhibitory effects of the antibody on MDA-MB-175 cells, e.g, essentially as described in Schaefer et al. *Oncogene* 15:1385-1394 (1997). According to this assay, MDA-MB-175 cells may treated with an anti-ErbB2 monoclonal antibody (10μg/mL) for 4 days and stained with crystal violet. Incubation with an anti-ErbB2 antibody may show a growth inhibitory effect on this cell line similar to that displayed by monoclonal antibody 2C4. In a further embodiment, exogenous HRG will not significantly reverse this inhibition. Preferably, the antibody will be able to inhibit cell proliferation of MDA-MB-175 cells to a greater extent than monoclonal antibody 4D5 (and optionally to a greater extent than monoclonal antibody 7F3), both in the presence and absence of exogenous HRG.

In one embodiment, the anti-ErbB2 antibody of interest may block heregulin dependent association of ErbB2 with ErbB3 in both MCF7 and SK-BR-3 cells as determined in a co-immunoprecipitation experiment such as that described in Example 2 substantially more effectively than monoclonal antibody 4D5, and preferably substantially more effectively than monoclonal antibody 7F3.

To identify growth inhibitory anti-ErbB2 antibodies, one may screen for antibodies which inhibit the growth of cancer cells which overexpress ErbB2. In one embodiment, the growth inhibitory antibody of choice is able to inhibit growth of SK-BR-3 cells in cell culture by about 20-100% and preferably by about 50-100% at an antibody concentration of about 0.5 to 30 μg/ml. To identify such antibodies, the SK-BR-3 assay described in U.S. Pat. No. 5,677,171 can be performed. According to this assay, SK-BR-3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin streptomycin. The SK-BR-3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 0.5 to 30 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SK-BR-3 cells by about 20-100% or about 50-100% may be selected as growth inhibitory antibodies.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake may be assessed relative to control. The preferred assay is the PI uptake assay using BT474 cells. According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection (Rockville, Md.)) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate monoclonal antibody. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed

with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake may be selected as cell death-inducing antibodies.

In order to select for antibodies which induce apoptosis, an annexin binding assay using BT474 cells is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the monoclonal antibody. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a DNA staining assay using BT474 cells is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFITLT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, or additionally, epitope mapping can be performed by methods known in the art (see, e.g. FIGS. **1**A and **1**B herein).

(ix) Immunoconjugates

The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. a small molecule toxin or an enzymatically active toxin of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, a maytansine (U.S. Pat. No. 5,208,020), a trichothene, and CC1065 are also contemplated herein.

In one preferred embodiment of the invention, the antibody is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody (Chari et al. *Cancer Research* 52: 127-131 (1992)) to generate a maytansinoid-antibody immunoconjugate.

Another immunoconjugate of interest comprises an anti-ErbB2 antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ_1^I (Hinman et al. *Cancer Research* 53: 3336-3342 (1993) and Lode et al. *Cancer Research* 58: 2925-2928 (1998)). See, also, U.S. Pat. Nos. 5,714,586; 5,712,374; 5,264,586; and 5,773,001 expressly incorporated herein by reference.

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), *momordica charantia* inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published Oct. 28, 1993.

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

A variety of radioactive isotopes are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al. *Cancer Research* 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the anti-ErbB2 antibody and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis.

In yet another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemo-

therapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as *serratia* protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuramimidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature,* 312: 604-608 (1984).

(xi) Other Antibody Modifications

Other modifications of the antibody are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences,* 16th edition, Oslo, A., Ed., (1980).

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA,* 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published Oct. 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19)1484 (1989).

III. Vectors, Host Cells and Recombinant Methods

The invention also provides isolated nucleic acid encoding the humanized anti-ErbB2 antibody, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody.

For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

The anti-ErbB2 antibody of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native anti-ErbB2 antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, 1pp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, a factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the anti-ErbB2 antibody.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

(iii) Selection Gene Component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the anti-ErbB2 antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity.

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding anti-ErbB2 antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., *Nature,* 282:39 (1979)). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics,* 85:12 (1977). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K lactis*. Van den Berg, *Bio/Technology,* 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer et al., *Bio/Technology,* 9:968-975 (1991).

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the anti-ErbB2 antibody nucleic acid. Promoters suitable for use with prokaryotic hosts include the phoA promoter, β-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the anti-ErbB2 antibody.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Anti-ErbB2 antibody transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Reyes et al., *Nature* 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the rous sarcoma virus long terminal repeat can be used as the promoter.

(v) Enhancer Element Component

Transcription of a DNA encoding the anti-ErbB2 antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the anti-ErbB2 antibody-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding anti-ErbB2 antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

(vii) Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella*, e.g., *Salmonella typhimurium, Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 Apr. 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-ErbB2 antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe; Kluyveromyces* hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa; Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated anti-ErbB2 antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR(CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TR1 cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for anti-ErbB2 antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

(viii) Culturing the Host Cells

The host cells used to produce the anti-ErbB2 antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(ix) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

IV. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or additionally, the composition may further comprise a chemotherapeutic agent, cytotoxic agent, cytokine, growth inhibitory agent, anti-hormonal agent, EGFR-targeted drug, anti-angiogenic agent, and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques, are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

V. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various diseases or disorders. Exemplary conditions or disorders include benign or malignant tumors; leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelic, inflammatory, angiogenic and immunologic disorders.

Generally, the disease or disorder to be treated is cancer. Examples of cancer to be treated herein include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

The cancer will generally comprise ErbB2-expressing cells, such that the anti-ErbB2 antibody herein is able to bind to the cancer. While the cancer may be characterized by overexpression of the ErbB2 receptor, the present application

further provides a method for treating cancer which is not considered to be an ErbB2-overexpressing cancer. To determine ErbB2 expression in the cancer, various diagnostic/prognostic assays are available. In one embodiment, ErbB2 overexpression may be analyzed by IHC, e.g. using the HERCEPTEST® (Dako). Parrafin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a ErbB2 protein staining intensity criteria as follows:

Score 0

no staining is observed or membrane staining is observed in less than 10% of tumor cells.

Score 1+

a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.

Score 2+

a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score 3+

a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

Those tumors with 0 or 1+ scores for ErbB2 overexpression assessment may be characterized as not overexpressing ErbB2, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing ErbB2.

Alternatively, or additionally, FISH assays such as the INFORM™ (sold by Ventana, Ariz.) or PATHVISION™ (Vysis, Ill.) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of ErbB2 overexpression in the tumor.

In one embodiment, the cancer will be one which expresses (and may overexpress) EGFR. Examples of cancers which may express/overexpress EGFR include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

The cancer to be treated herein may be one characterized by excessive activation of an ErbB receptor, e.g. EGFR. Such excessive activation may be attributable to overexpression or increased production of the ErbB receptor or an ErbB ligand. In one embodiment of the invention, a diagnostic or prognostic assay will be performed to determine whether the patient's cancer is characterized by excessive activation of an ErbB receptor. For example, ErbB gene amplification and/or overexpression of an ErbB receptor in the cancer may be determined. Various assays for determining such amplification/overexpression are available in the art and include the IHC, FISH and shed antigen assays described above. Alternatively, or additionally, levels of an ErbB ligand, such as TGF-α, in or associated with the tumor may be determined according to known procedures. Such assays may detect protein and/or nucleic acid encoding it in the sample to be tested. In one embodiment, ErbB ligand levels in the tumor may be determined using immunohistochemistry (IHC); see, for example, Scher et al. *Clin. Cancer Research* 1:545-550 (1995). Alternatively, or additionally, one may evaluate levels of ErbB ligand-encoding nucleic acid in the sample to be tested; e.g. via FISH, southern blotting, or PCR techniques.

Moreover, ErbB receptor or ErbB ligand overexpression or amplification may be evaluated using an in vivo diagnostic assay, e.g. by administering a molecule (such as an antibody) which binds the molecule to be detected and is tagged with a detectable label (e.g. a radioactive isotope) and externally scanning the patient for localization of the label.

Where the cancer to be treated is hormone independent cancer, expression of the hormone (e.g. androgen) and/or its cognate receptor in the tumor may be assessed using any of the various assays available, e.g. as described above. Alternatively, or additionally, the patient may be diagnosed as having hormone independent cancer in that they no longer respond to anti-androgen therapy.

In certain embodiments, an immunoconjugate comprising the anti-ErbB2 antibody conjugated with a cytotoxic agent is administered to the patient. Preferably, the immunoconjugate and/or ErbB2 protein to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate in killing the cancer cell to which it binds. In a preferred embodiment, the cytotoxic agent targets or interferes with nucleic acid in the cancer cell. Examples of such cytotoxic agents include maytansinoids, calicheamicins, ribonucleases and DNA endonucleases.

The anti-ErbB2 antibodies or immunoconjugates are administered to a human patient in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

Other therapeutic regimens may be combined with the administration of the anti-ErbB2 antibody. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities.

In one preferred embodiment, the patient is treated with two different anti-ErbB2 antibodies. For example, the patient may be treated with a first anti-ErbB2 antibody which blocks ligand activation of an ErbB receptor or an antibody having a biological characteristic of monoclonal antibody 2C4 as well as a second anti-ErbB2 antibody which is growth inhibitory (e.g. HERCEPTIN®) or an anti-ErbB2 antibody which induces apoptosis of an ErbB2-overexpressing cell (e.g. 7C2, 7F3 or humanized variants thereof). Preferably such combined therapy results in a synergistic therapeutic effect. One may, for instance, treat the patient with HERCEPTIN® and thereafter treat with rhuMAb 2C4, e.g. where the patient does not respond to HERCEPTIN® therapy. In another embodiment, the patient may first be treated with rhuMAb 2C4 and then receive HERCEPTIN® therapy. In yet a further embodiment, the patient may be treated with both rhuMAb 2C4 and HERCEPTIN® simultaneously.

It may also be desirable to combine administration of the anti-ErbB2 antibody or antibodies, with administration of an antibody directed against another tumor associated antigen. The other antibody in this case may, for example, bind to EGFR, ErbB3, ErbB4, or vascular endothelial growth factor (VEGF).

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB2 antibody (or antibodies) and one or more chemotherapeutic agents or growth inhibitory agents, including coadministra-

tion of cocktails of different chemotherapeutic agents. Preferred chemotherapeutic agents include taxanes (such as paclitaxel and docetaxel) and/or anthracycline antibiotics. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkins, Baltimore, Md. (1992).

The antibody may be combined with an anti-hormonal compound; e.g., an anti-estrogen compound such as tamoxifen; an anti-progesterone such as onapristone (see, EP 616 812); or an anti-androgen such as flutamide, in dosages known for such molecules. Where the cancer to be treated is hormone independent cancer, the patient may previously have been subjected to anti-hormonal therapy and, after the cancer becomes hormone independent, the anti-ErbB2 antibody (and optionally other agents as described herein) may be administered to the patient.

Sometimes, it may be beneficial to also coadminister a cardioprotectant (to prevent or reduce myocardial dysfunction associated with the therapy) or one or more cytokines to the patient. One may also coadminister an EGFR-targeted drug or an anti-angiogenic agent. In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

The anti-ErbB2 antibodies herein may also be combined with an EGFR-targeted drug such as those discussed above in the definitions section resulting in a complementary, and potentially synergistic, therapeutic effect.

Suitable dosages for any of the above coadministered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and anti-ErbB2 antibody.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The preferred dosage of the antibody will be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, e.g. about six doses of the anti-ErbB2 antibody). An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the anti-ErbB2 antibody. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Aside from administration of the antibody protein to the patient, the present application contemplates administration of the antibody by gene therapy. Such administration of nucleic acid encoding the antibody is encompassed by the expression "administering a therapeutically effective amount of an antibody". See, for example, WO96/07321 published Mar. 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells; in vivo and ex vivo. For in vivo delivery the nucleic acid is injected directly into the patient, usually at the site where the antibody is required. For ex vivo treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, e.g. U.S. Pat. Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for ex vivo delivery of the gene is a retrovirus.

The currently preferred in vivo nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., *J. Biol. Chem.* 262:4429-4432 (1987); and Wagner et al., *Proc. Natl. Acad. Sci. USA* 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson et al., *Science* 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label or package insert indicates that the composition is used for treating the condition of choice, such as cancer. In one embodiment, the label or package inserts indicates that the composition com-

prising the antibody which binds ErbB2 can be used to treat cancer which expresses an ErbB receptor selected from the group consisting of epidermal growth factor receptor (EGFR), ErbB3 and ErbB4, preferably EGFR. In addition, the label or package insert may indicate that the patient to be treated is one having cancer characterized by excessive activation of an ErbB receptor selected from EGFR, ErbB3 or ErbB4. For example, the cancer may be one which overexpresses one of these receptors and/or which overexpresses an ErbB ligand (such as TGF-α). The label or package insert may also indicate that the composition can be used to treat cancer, wherein the cancer is not characterized by overexpression of the ErbB2 receptor. For example, whereas the present package insert for HERCEPTIN® indicates that the antibody is used to treat patients with metastatic breast cancer whose tumors overexpress the ErbB2 protein, the package insert herein may indicate that the antibody or composition is used to treat cancer regardless of the extent of ErbB2 overexpression. In other embodiments, the package insert may indicate that the antibody or composition can be used to treat breast cancer (e.g. metastatic breast cancer); hormone independent cancer; prostate cancer, (e.g. androgen independent prostate cancer); lung cancer (e.g. non-small cell lung cancer); colon, rectal or colorectal cancer; or any of the other diseases or disorders disclosed herein. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises a first antibody which binds ErbB2 and inhibits growth of cancer cells which overexpress ErbB2; and (b) a second container with a composition contained therein, wherein the composition comprises a second antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor. The article of manufacture in this embodiment of the invention may further comprises a package insert indicating that the first and second antibody compositions can be used to treat cancer. Moreover, the package insert may instruct the user of the composition (comprising an antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor) to combine therapy with the antibody and any of the adjunct therapies described in the preceding section (e.g. a chemotherapeutic agent, an EGFR-targeted drug, an anti-angiogenic agent, an anti-hormonal compound, a cardioprotectant and/or a cytokine). Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

VII. Non-Therapeutic Uses for the Anti-ErbB2 Antibody

The antibodies (e.g. the humanized anti-ErbB2 antibodies) of the invention have further non-therapeutic applications.

For example, the antibodies may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody is contacted with a sample containing the ErbB2 protein (or fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the ErbB2 protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the ErbB2 protein from the antibody.

Anti-ErbB2 antibodies may also be useful in diagnostic assays for ErbB2 protein, e.g., detecting its expression in specific cells, tissues, or serum.

For diagnostic applications, the antibody typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S, ^{14}C, ^{125}I, ^{3}H, and ^{131}I. The antibody can be labeled with the radioisotope using the techniques described in *Current Protocols in Immunology*, Volumes 1 and 2, Coligen et al., Ed. Wiley-Interscience, New York, N.Y., Pubs. (1991) for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in *Current Protocols in Immunology*, supra, for example. Fluorescence can be quantified using a fluorimeter.

(c) Various enzyme-substrate labels are available and U.S. Pat. No. 4,275,149 provides a review of some of these. The enzyme generally catalyzes a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (e.g., firefly luciferase and bacterial luciferase; U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan et al., Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in *Methods in Enzym.* (ed J. Langone & H. Van Vunakis), Academic press, New York, 73:147-166 (1981).

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (e.g., orthophenylene diamine (OPD) or 3,3', 5,5'-tetramethyl benzidine hydrochloride (TMB));

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and

(iii) β-D-galactosidase (β-D-Gal) with a chromogenic substrate (e.g., p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Pat. Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or vice versa. Biotin binds selectively to avidin and thus, the label can

be conjugated with the antibody in this indirect manner. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (e.g., digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (e.g., anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-ErbB2 antibody need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the ErbB2 antibody.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp. 147-158 (CRC Press, Inc. 1987).

For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

The antibodies may also be used for in vivo diagnostic assays. Generally, the antibody is labeled with a radio nuclide (such as ^{111}In, ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^{3}H, ^{32}P or ^{35}S) so that the tumor can be localized using immunoscintiography. As a matter of convenience, the antibodies of the present invention can be provided in a kit, i.e., a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (e.g., a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (e.g., a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

VIII. Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209, USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1

Production and Characterization of Monoclonal Antibody 2C4

The murine monoclonal antibodies 2C4, 7F3 and 4D5 which specifically bind the extracellular domain of ErbB2 were produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990). Briefly, NIH 3T3/HER2-3$_{400}$ cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al *Proc. Natl. Acad. Sci. (USA)* 84:7159-7163 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653.

Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation.

The ErbB2 epitopes bound by monoclonal antibodies 4D5, 7F3 and 2C4 were determined by competitive binding analysis (Fendly et al. *Cancer Research* 50:1550-1558 (1990)). Cross-blocking studies were done on antibodies by direct fluorescence on intact cells using the PANDEX™ Screen Machine to quantitate fluorescence. Each monoclonal antibody was conjugated with fluorescein isothiocyanate (FITC), using established procedures (Wofsy et al. *Selected Methods in Cellular Immunology*, p. 287, Mishel and Schiigi (eds.) San Francisco: W.J. Freeman Co. (1980)). Confluent monolayers of NIH 3T3/HER2-3$_{400}$ cells were trypsinized, washed once, and resuspended at 1.75×10^6 cell/ml in cold PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3. A final concentration of 1% latex particles (IDC, Portland, Oreg.) was added to reduce clogging of the PANDEX™ plate membranes. Cells in suspension, 20 μl, and 20 μl of purified monoclonal antibodies (100 μg/ml to 0.1 μg/ml) were added to the PANDEX™ plate wells and incubated on ice for 30 minutes. A predetermined dilution of FITC-labeled monoclonal antibodies in 20 μl was added to each well, incubated for 30 minutes, washed, and the fluorescence was quantitated by the PANDEX™. Monoclonal antibodies were considered to share an epitope if each blocked binding of the other by 50% or greater in comparison to an irrelevant monoclonal antibody control. In this experiment, monoclonal antibodies 4D5, 7F3 and 2C4 were assigned epitopes I, G/F and F, respectively.

The growth inhibitory characteristics of monoclonal antibodies 2C4, 7F3 and 4D5 were evaluated using the breast tumor cell line, SK-BR-3 (see Hudziak et al. *Molec. Cell. Biol.* 9(3):1165-1172 (1989)). Briefly, SK-BR-3 cells were detached by using 0.25% (vol/vol) trypsin and suspended in complete medium at a density of 4×10^5 cells per ml. Aliquots of 100 μl (4×10^4 cells) were plated into 96-well microdilution plates, the cells were allowed to adhere, and 100 μl of media alone or media containing monoclonal antibody (final concentration 5 μg/ml) was then added. After 72 hours, plates were washed twice with PBS (pH 7.5), stained with crystal violet (0.5% in methanol), and analyzed for relative cell proliferation as described in Sugarman et al. *Science* 230:943-945 (1985). Monoclonal antibodies 2C4 and 7F3 inhibited SK-BR-3 relative cell proliferation by about 20% and about 38%, respectively, compared to about 56% inhibition achieved with monoclonal antibody 4D5.

Monoclonal antibodies 2C4, 4D5 and 7F3 were evaluated for their ability to inhibit HRG-stimulated tyrosine phosphorylation of proteins in the M_r 180,000 range from whole-cell lysates of MCF7 cells (Lewis et al. *Cancer Research* 56:1457-1465 (1996)). MCF7 cells are reported to express all known ErbB receptors, but at relatively low levels. Since ErbB2, ErbB3, and ErbB4 have nearly identical molecular

sizes, it is not possible to discern which protein is becoming tyrosine phosphorylated when whole-cell lysates are evaluated by Western blot analysis.

However, these cells are ideal for HRG tyrosine phosphorylation assays because under the assay conditions used, in the absence of exogenously added HRG, they exhibit low to undetectable levels of tyrosine phosphorylation proteins in the M_r 180,000 range.

MCF7 cells were plated in 24-well plates and monoclonal antibodies to ErbB2 were added to each well and incubated for 30 minutes at room temperature; then rHRGβ1$_{177-244}$ was added to each well to a final concentration of 0.2 nM, and the incubation was continued for 8 minutes. Media was carefully aspirated from each well, and reactions were stopped by the addition of 100 μl of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 μl) was electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (4G10, from UBI, used at 1 μg/ml) immunoblots were developed, and the intensity of the predominant reactive band at M_r ~180,000 was quantified by reflectance densitometry, as described previously (Holmes et al. *Science* 256:1205-1210 (1992); Sliwkowski et al. *J. Biol. Chem.* 269:14661-14665 (1994)).

Monoclonal antibodies 2C4, 7F3, and 4D5, significantly inhibited the generation of a HRG-induced tyrosine phosphorylation signal at M_r 180,000. In the absence of HRG, none of these antibodies were able to stimulate tyrosine phosphorylation of proteins in the M_r 180,000 range. Also, these antibodies do not cross-react with EGFR (Fendly et al. *Cancer Research* 50:1550-1558 (1990)), ErbB3, or ErbB4. Antibodies 2C4 and 7F3 significantly inhibited HRG stimulation of p180 tyrosine phosphorylation to <25% of control. Monoclonal antibody 4D5 was able to block HRG stimulation of tyrosine phosphorylation by ~50%. FIG. **2**A shows dose-response curves for 2C4 or 7F3 inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry. Evaluation of these inhibition curves using a 4-parameter fit yielded an IC_{50} of 2.8±0.7 nM and 29.0±4.1 nM for 2C4 and 7F3, respectively.

Inhibition of HRG binding to MCF7 breast tumor cell lines by anti-ErbB2 antibodies was performed with monolayer cultures on ice in a 24-well-plate format (Lewis et al. *Cancer Research* 56:1457-1465 (1996)). Anti-ErbB2 monoclonal antibodies were added to each well and incubated for 30 minutes. ^{125}I-labeled rHRGβ1$_{177-224}$ (25 pm) was added, and the incubation was continued for 4 to 16 hours. FIG. **2**B provides dose-response curves for 2C4 or 7F3 inhibition of HRG binding to MCF7 cells. Varying concentrations of 2C4 or 7F3 were incubated with MCF7 cells in the presence of ^{125}I-labeled rHRGβ1, and the inhibition curves are shown in FIG. **2**B. Analysis of these data yielded an IC_{50} of 2.4±0.3 nM and 19.0±7.3 nM for 2C4 and 7F3, respectively. A maximum inhibition of ~74% for 2C4 and 7F3 were in agreement with the tyrosine phosphorylation data.

To determine whether the effect of the anti-ErbB2 antibodies observed on MCF7 cells was a general phenomenon, human tumor cell lines were incubated with 2C4 or 7F3 and the degree of specific ^{125}I-labeled rHRGβ1 binding was determined (Lewis et al. *Cancer Research* 56:1457-1465 (1996)). The results from this study are shown in FIG. **3**. Binding of ^{121}I-labeled rHRGβ1 could be significantly inhibited by either 2C4 or 7F3 in all cell lines, with the exception of the breast cancer cell line MDA-MB-468, which has been reported to express little or no ErbB2. The remaining cell lines are reported to express ErbB2, with the level of ErbB2 expression varying widely among these cell lines. In fact, the range of ErbB2 expression in the cell lines tested varies by more than 2 orders of magnitude. For example, BT-20, MCF7, and Caov3 express ~10^4 ErbB2 receptors/cell, whereas BT-474 and SK-BR-3 express ~10^6 ErbB2 receptors/cell. Given the wide range of ErbB2 expression in these cells and the data above, it was concluded that the interaction between ErbB2 and ErbB3 or ErbB4, was itself a high-affinity interaction that takes place on the surface of the plasma membrane.

The growth inhibitory effects of monoclonal antibodies 2C4 and 4D5 on MDA-MB-175 and SK-BR-3 cells in the presence or absence of exogenous rHRGβ1 was assessed (Schaefer et al. *Oncogene* 15:1385-1394 (1997)). ErbB2 levels in MDA-MB-175 cells are 4-6 times higher than the level found in normal breast epithelial cells and the ErbB2-ErbB4 receptor is constitutively tyrosine phosphorylated in MDA-MB-175 cells. MDA-MB-175 cells were treated with an anti-ErbB2 monoclonal antibodies 2C4 and 4D5 (10 μg/mL) for 4 days. In a crystal violet staining assay, incubation with 2C4 showed a strong growth inhibitory effect on this cell line (FIG. **4**A). Exogenous HRG did not significantly reverse this inhibition. On the other hand 2C4 revealed no inhibitory effect on the ErbB2 overexpressing cell line SK-BR-3 (FIG. **4**B). Monoclonal antibody 2C4 was able to inhibit cell proliferation of MDA-MB-175 cells to a greater extent than monoclonal antibody 4D5, both in the presence and absence of exogenous HRG. Inhibition of cell proliferation by 4D5 is dependent on the ErbB2 expression level (Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993)). A maximum inhibition of 66% in SK-BR-3 cells could be detected (FIG. **4**B). However this effect could be overcome by exogenous HRG.

EXAMPLE 2

HRG Dependent Association of ErbB2 with ErbB3 is Blocked by Monoclonal Antibody 2C4

The ability of ErbB3 to associate with ErbB2 was tested in a co-immunoprecipitation experiment. 1.0×10^6 MCF7 or SK-BR-3 cells were seeded in six well tissue culture plates in 50:50 DMEM/Ham's F12 medium containing 10% fetal bovine serum (FBS) and 10 mM HEPES, pH 7.2 (growth medium), and allowed to attach overnight. The cells were starved for two hours in growth medium without serum prior to beginning the experiment

The cells were washed briefly with phosphate buffered saline (PBS) and then incubated with either 100 nM of the indicated antibody diluted in 0.2% w/v bovine serum albumin (BSA), RPMI medium, with 10 mM HEPES, pH 7.2 (binding buffer), or with binding buffer alone (control). After one hour at room temperature, HRG was added to a final concentration of 5 nM to half the wells (+). A similar volume of binding buffer was added to the other wells (−). The incubation was continued for approximately 10 minutes.

Supernatants were removed by aspiration and the cells were lysed in RPMI, 10 mM HEPES, pH 7.2, 1.0% v/v TRITON X-100™, 1.0% w/v CHAPS (lysis buffer), containing 0.2 mM PMSF, 10 μg/ml leupeptin, and 10 TU/ml aprotinin. The lysates were cleared of insoluble material by centrifugation.

ErbB2 was immunoprecipitated using a monoclonal antibody covalently coupled to an affinity gel (Affi-Prep 10, Bio-Rad). This antibody (Ab-3, Oncogene Sciences) recognizes a cytoplasmic domain epitope. Immunoprecipitation was performed by adding 10 μl of gel slurry containing approximately 8.5 μg of immobilized antibody to each lysate,

and the samples were allowed to mix at room temperature for two hours. The gels were then collected by centrifugation. The gels were washed batchwise three times with lysis buffer to remove unbound material. SDS sample buffer was then added and the samples were heated briefly in a boiling water bath.

Supernatants were run on 4-12% polyacrylamide gels and electroblotted onto nitrocellulose membranes. The presence of ErbB3 was assessed by probing the blots with a polyclonal antibody against a cytoplasmic domain epitope thereof (c-17, Santa Cruz Biotech). The blots were visualized using a chemiluminescent substrate (ECL, Amersham)

As shown in the control lanes of FIGS. **5**A and **5**B, for MCF7 and SK-BR-3 cells, respectively, ErbB3 was present in an ErbB2 immunoprecipitate only when the cells were stimulated with HRG. If the cells were first incubated with monoclonal antibody 2C4, the ErbB3 signal was abolished in MCF7 cells (FIG. **5**A, lane 2C4+) or substantially reduced in SK-BR-3 cells (FIG. **5**B, lane 2C4+). As shown in FIGS. **5**A-B, monoclonal antibody 2C4 blocks heregulin dependent association of ErbB3 with ErbB2 in both MCF7 and SK-BR-3 cells substantially more effectively than HERCEPTIN®. Preincubation with HERCEPTIN® decreased the ErbB3 signal in MCF7 lysates but had little or no effect on the amount of ErbB3 co-precipitated from SK-BR-3 lysates. Preincubation with an antibody against the EGF receptor (Ab-1, Oncogene Sciences) had no effect on the ability of ErbB3 to co-immunoprecipitate with ErbB2 in either cell line.

EXAMPLE 3

Humanized 2C4 Antibodies

The variable domains of murine monoclonal antibody 2C4 were first cloned into a vector which allows production of a mouse/human chimeric Fab fragment. Total RNA was isolated from the hybridoma cells using a Stratagene RNA extraction kit following manufacturer's protocols. The variable domains were amplified by RT-PCR, gel purified, and inserted into a derivative of a pUC119-based plasmid containing a human kappa constant domain and human C_H1 domain as previously described (Carter et al. *PNAS* (*USA*) 89:4285 (1992); and U.S. Pat. No. 5,821,337). The resultant plasmid was transformed into *E. coli* strain 16C9 for expression of the Fab fragment. Growth of cultures, induction of protein expression, and purification of Fab fragment were as previously described (Werther et al. *J. Immunol.* 157:4986-4995 (1996); Presta et al. *Cancer Research* 57: 4593-4599 (1997)).

Purified chimeric 2C4 Fab fragment was compared to the murine parent antibody 2C4 with respect to its ability to inhibit ^{125}I-HRG binding to MCF7 cells and inhibit rHRG activation of p180 tyrosine phosphorylation in MCF7 cells. As shown in FIG. **6**A, the chimeric 2C4 Fab fragment is very effective in disrupting the formation of the high affinity ErbB2-ErbB3 binding site on the human breast cancer cell line, MCF7. The relative IC_{50} value calculated for intact murine 2C4 is 4.0±0.4 nM, whereas the value for the Fab fragment is 7.7±1.1 nM. As illustrated in FIG. **6**B, the monovalent chimeric 2C4 Fab fragment is very effective in disrupting HRG-dependent ErbB2-ErbB3 activation. The IC_{50} value calculated for intact murine monoclonal antibody 2C4 is 6.0±2 nM, whereas the value for the Fab fragment is 15.0±2 nM.

DNA sequencing of the chimeric clone allowed identification of the CDR residues (Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) (FIGS. **7**A and B). Using oligonucleotide site-directed mutagenesis, all six of these CDR regions were introduced into a complete human framework (V_L kappa subgroup I and V_H subgroup III) contained on plasmid VX4 as previously described (Presta et al., *Cancer Research* 57: 4593-4599 (1997)). Protein from the resultant "CDR-swap" was expressed and purified as above. Binding studies were performed to compare the two versions. Briefly, a NUNC MAXISORP™ plate was coated with 1 microgram per ml of ErbB2 extracellular domain (ECD; produced as described in WO 90/14357) in 50 mM carbonate buffer, pH 9.6, overnight at 4° C., and then blocked with ELISA diluent (0.5% BSA, 0.05% polysorbate 20, PBS) at room temperature for 1 hour. Serial dilutions of samples in ELISA diluent were incubated on the plates for 2 hours. After washing, bound Fab fragment was detected with biotinylated murine anti-human kappa antibody (ICN 634771) followed by streptavidin-conjugated horseradish peroxidase (Sigma) and using 3,3',5,5'-tetramethyl benzidine (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) as substrate. Absorbance was read at 450 nm. As shown in FIG. **8**A, all binding was lost on construction of the CDR-swap human Fab fragment.

To restore binding of the humanized Fab, mutants were constructed using DNA from the CDR-swap as template. Using a computer generated model (FIG. **9**), these mutations were designed to change human framework region residues to their murine counterparts at positions where the change might affect CDR conformations or the antibody-antigen interface. Mutants are shown in Table 2.

TABLE 2

Designation of Humanized 2C4 FR Mutations

Mutant no.	Framework region (FR) substitutions
560	ArgH71Val
561	AspH73Arg
562	ArgH71Val, AspH73Arg
568	ArgH71Val, AspH73Arg, AlaH49Gly
569	ArgH71Val, AspH73Arg, PheH67Ala
570	ArgH71Val, AspH73Arg, AsnH76Arg
571	ArgH71Val, AspH73Arg, LeuH78Val
574	ArgH71Val, AspH73Arg, IleH69Leu
56869	ArgH71Val, AspH73Arg, AlaH49Gly, PheH67Ala

Binding curves for the various mutants are shown in FIGS. **8**A-C. Humanized Fab version 574, with the changes ArgH71Val, AspH73Arg and IleH69Leu, appears to have binding restored to that of the original chimeric 2C4 Fab fragment. Additional FR and/or CDR residues, such as L2, L54, L55, L56, H35 and/or H48, may be modified (e.g. substituted as follows—IleL2Thr; ArgL54Leu; TyrL55Glu; ThrL56Ser; AspH35Ser; and ValH48Ile) in order to further refine or enhance binding of the humanized antibody. Alternatively, or additionally, the humanized antibody may be

affinity matured (see above) in order to further improve or refine its affinity and/or other biological activities.

Humanized 2C4 version 574 was affinity matured using a phage-display method. Briefly, humanized 2C4.574 Fab was cloned into a phage display vector as a geneIII fusion. When phage particles are induced by infection with M13KO7 helper phage, this fusion allows the Fab to be displayed on the N-terminus of the phage tail-fiber protein, geneIII (Baca et al. *J Biol. Chem.* 272:10678 (1997)).

Individual libraries were constructed for each of the 6 CDRs identified above. In these libraries, the amino acids in the CDRs which were identified using a computer generated model (FIG. **9**) as being potentially significant in binding to ErbB2 were randomized using oligos containing "NNS" as their codons. The libraries were then panned against ErbB2 ECD coated on NUNC MAXISORP™ plates with 3% dry milk in PBS with 0.2% TWEEN 20® (MPBST) used in place of all blocking solutions. In order to select for phage with affinities higher than that of 2C4.574, in panning rounds 3, 4, and 5, soluble ErbB2 ECD or soluble Fab 2C4.574 was added during the wash steps as competitor. Wash times were extended to 1 hour at room temperature.

After 5 rounds of panning, individual clones were again analyzed by phage-ELISA. Individual clones were grown in Costar 96-well U-bottomed tissue culture plates, and phage were induced by addition of helper phage. After overnight growth, *E. coli* cells were pelleted, and the phage-containing supernates were transfered to 96-well plates where the phage were blocked with MPBST for 1 hr at room temperature. NUNC MAXISORP™ plates coated with ErbB2 ECD were also blocked with MPBST as above. Blocked phage were incubated on the plates for 2 hours. After washing, bound phage were detected using horseradish-peroxidase-conjugated anti-M13 monoclonal antibody (Amersham Pharmacia Biotech, Inc. 27-9421-01) diluted 1:5000 in MPBST, followed by 3,3',5,5',-tetramethyl benzidine as substrate. Absorbance was read at 450 nm.

The 48 clones from each library which gave the highest signals were DNA sequenced. Those clones whose sequences occurred the most frequently were subcloned into the vector described above which allows expression of soluble Fabs. These Fabs were induced, proteins purified and the purified Fabs were analyzed for binding by ELISA as described above and the binding was compared to that of the starting humanized 2C4.574 version.

After interesting mutations in individual CDRs were identified, additional mutants which were various combinations of these were constructed and tested as above. Mutants which gave improved binding relative to 574 are described in Table 3.

TABLE 3

Designation of mutants derived from affinity maturation of 2C4.574

Mutant Name	Change from 574	Mutant/574*
H3.A1	serH99trp, metH34leu	0.380
L2.F5	serL50trp, tyrL53gly, metH34leu	0.087
H1.3.B3	thrH28gln, thrH30ser, metH34leu	0.572
L3.G6	tyrL92pro, ileL93lys, metH34leu	0.569
L3.G11	tyrL92ser, ileL93arg, tyrL94gly, metH34leu	0.561
L3.29	tyrL92phe, tyrL96asn, metH34leu	0.552
L3.36	tyrL92phe, tyrL94leu, tyrL96pro, metH34leu	0.215
654	serL50trp, metH34leu	0.176
655	metH34ser	0.542
659	serL50trp, metH34ser	0.076
L2.F5.H3.A1	serL50trp, tyrL53gly, metH34leu, serH99trp	0.175
L3G6.H3.A1	tyrL92pro, ileL93lys, metH34leu, serH99trp	0.218
H1.3.B3.H3.A1	thrH28gln, thrH30ser, metH34leu, serH99trp	0.306
L3.G11.H3.A1	tyrL92ser, ileL93arg, tyrL94gly, metH34leu, serH99trp	0.248
654.H3.A1	serL50trp, metH34leu, serH99trp	0.133
654.L3.G6	serL50trp, metH34leu, tyrL92pro, ileL93lys	0.213
654.L3.29	serL50trp, metH34leu, tyrL92phe, tyrL96asn	0.236
654.L3.36	serL50trp, metH35leu, tyrL92phe, tyrL94leu, tyrL96pro	0.141

*Ratio of the amount of mutant needed to give the mid-OD of the standard curve to the amount of 574 needed to give the mid-OD of the standard curve in an Erb2-ECD ELISA. A number less than 1.0 indicates that the mutant binds Erb2 better than 574 binds.

The following mutants have also been constructed, and are currently under evaluation:

659.L3.G6	serL50trp, metH34ser, tyrL92pro, ileL93lys
659.L3.G11	serL50trp, metH34ser, tyrL92ser, ileL93arg, tyrL94gly
659.L3.29	serL50trp, metH34ser, tyrL92phe, tyrL96asn
659.L3.36	serL50trp, metH34ser, tyrL92phe, tyrL94leu, tyrL96pro
L2F5.L3G6	serL50trp, tyrL53gly, metH34leu, tyrL92pro, ileL93lys
L2F5.L3G11	serL50trp, tyrL53gly, metH34leu, tyrL92ser, ileL93arg, tyrL94gly
L2F5.L29	serL50trp, tyrL53gly, metH34leu, tyrL92phe, tyrL96asn
L2F5.L36	serL50trp, tyrL53gly, metH34leu, tyrL92phe, tyrL94leu, tyrL96pro
L2F5.L3G6.655	serL50trp, tyrL53gly, metH35ser, tyrL92pro, ileL93lys
L2F5.L3G11.655	serL50trp, tyrL53gly, metH34ser, tyrL92ser, ileL93arg, tyrL94gly

-continued

L2F5.L29.655	serL50trp, tyrL53gly, metH34ser, tyrL92phe, tyrL96asn
L2F5.L36.655	serL50trp, tyrL53gly, metH34ser, tyrL92phe, tyrL94leu, tyrL96pro

The following mutants, suggested by a homology scan, are currently being constructed:

678	thrH30ala
679	thrH30ser
680	lysH64arg
681	leuH96val
682	thrL97ala
683	thrL97ser
684	tyrL96phe
685	tyrL96ala
686	tyrL91phe
687	thrL56ala
688	glnL28ala
689	glnL28glu

The preferred amino acid at H34 would be methionine. A change to leucine might be made if there were found to be oxidation at this position.

AsnH52 and asnH53 were found to be strongly preferred for binding. Changing these residues to alanine or aspartic acid dramatically decreased binding.

An intact antibody comprising the variable light and heavy domains of humanized version 574 with a human IgG1 heavy chain constant region has been prepared (see U.S. Pat. No. 5,821,337). The intact antibody is produced by Chinese Hamster Ovary (CHO) cells. That molecule is designated rhuMAb 2C4 herein.

EXAMPLE 4

Monoclonal Antibody 2C4 Blocks EGF, TGF-α or HRG Mediated Activation of MAPK

Many growth factor receptors signal through the mitogen-activated protein kinase (MAPK) pathway. These dual specificity kinases are one of the key endpoints in signal transduction pathways that ultimately triggers cancer cells to divide. The ability of monoclonal antibody 2C4 or HERCEPTIN® to inhibit EGF, TGF-α or HRG activation of MAPK was assessed in the following way.

MCF7 cells (10^5 cells/well) were plated in serum containing media in 12-well cell culture plates. The next day, the cell media was removed and fresh media containing 0.1% serum was added to each well. This procedure was then repeated the following day and prior to assay the media was replaced with serum-free binding buffer (Jones et al. *J. Biol. Chem.* 273: 11667-74 (1998); and Schaefer et al. *J. Biol. Chem.* 274:859-66 (1999)). Cells were allowed to equilibrate to room temperature and then incubated for 30 minutes with 0.5 mL of 200 nM HERCEPTIN® or monoclonal antibody 2C4. Cells were then treated with 1 nM EGF, 1 nM TGF-α or 0.2 nM HRG for 15 minutes. The reaction was stopped by aspirating the cell medium and then adding 0.2 mL SDS-PAGE sample buffer containing 1% DTT. MAPK activation was assessed by Western blotting using an anti-active MAPK antibody (Promega) as described previously (Jones et al. *J. Biol. Chem.* 273:11667-74 (1998)).

As shown in FIG. **10**, monoclonal antibody 2C4 significantly blocks EGF, TGF-α and HRG mediated activation of MAPK to a greater extent than HERCEPTIN®. These data suggest that monoclonal antibody 2C4 binds to a surface of ErbB2 that is used for its association with either EGFR or ErbB3 and thus prevents the formation of the signaling receptor complex.

Monoclonal antibody 2C4 was also shown to inhibit heregulin (HRG)-dependent Akt activation. Activation of the PI3 kinase signal transduction pathway is important for cell survival (Carraway et al. *J. Biol. Chem.* 270: 7111-6 (1995)). In tumor cells, PI3 kinase activation may play a role in the invasive phenotype (Tan et al. *Cancer Reearch.* 59: 1620-1625, (1999)). The survival pathway is primarily mediated by the serine/threonine kinase AKT (Bos et al. *Trends Biochem Sci.* 20: 441-442 (1995). Complexes formed between ErbB2 and either ErbB3 or EGFR can initiate these pathways in response to heregulin or EGF, respectively (Olayioye et al. *Mol. & Cell. Biol.* 18: 5042-51 (1998); Karunagaran et al., *EMBO Journal.* 15: 254-264 (1996); and Krymskaya et al. *Am. J. Physiol.* 276: L246-55 (1999)). Incubation of MCF7 breast cancer cells with 2C4 inhibits heregulin-mediated AKT activation. Moreover, the basal level of AKT activation present in the absence of heregulin addition is further reduced by the addition of 2C4. These data suggest that 2C4 may inhibit ErbB ligand-activation of PI3 kinase and that this inhibition may lead to apoptosis. The increased sensitivity to apoptosis may manifest in a greater sensitivity of tumor cells to the toxic effects of chemotherapy.

Thus, monoclonal antibody 2C4 inhibits ligand initiated ErbB signaling through two major signal transduction pathways—MAP Kinase (a major proliferative pathway) and PI3 kinase (a major survival/anti-apoptotic pathway).

EXAMPLE 5

Combination of Monoclonal Antibody 2C4 and HERCEPTIN® In Vivo

A xenograft model using the lung adenocarcinoma cell line, Calu-3, was used to assess the efficacy of anti-HER2 monoclonal antibodies, either alone or in combination, to suppress tumor growth. Female NCR nude mice were inoculated subcutaneously with 20×10^6 cells in 0.1 mL. Tumor measurements were taken twice per week and when tumor nodules reached a volume of 100 mm^3, animals were randomized to 7 treatment groups. The treatment groups were:

(a) control monoclonal antibody, MAb 1766;
(b) HERCEPTIN®, 10 mg/kg;
(c) monoclonal antibody 7C2, 10 mg/kg;
(d) monoclonal antibody 2C4, 10 mg/kg;
(e) HERCEPTIN® and 7C2, each at 10 mg/kg;
(f) HERCEPTIN® and 2C4, each at 10 mg/kg; and
(g) Monoclonal antibodies 2C4 and 7C2, each at 10 mg/kg.

Animals were treated twice per week until day 24. Tumor volumes were measured twice per week until day 38.

As shown in the bar graph in FIG. **11**, treatment of the Calu-3 tumor-bearing mice with 2C4 or HERCEPTIN® significantly inhibited the growth of the tumors. The combination of HERCEPTIN® and 2C4 or HERCEPTIN® and 7C2 was superior to either monoclonal antibody administered alone.

EXAMPLE 6

Treating Colorectal Cancer with Monoclonal Antibody 2C4

Human colorectal cell lines such as HCA-7, LS 174T or CaCo-2 are implanted subcutaneously in athymic nude mice as described in Sheng et al. *J. Clin. Invest.* 99:2254-2259 (1997). Once tumors are established to about 100 mm^3 in volume, groups of animals are treated with 10-50 mg/kg of monoclonal antibody 2C4 administered twice weekly by injection in the intraperitoneal cavity. Monoclonal antibody 2C4 suppresses growth of colorectal xenografts in vivo.

EXAMPLE 7

Treating Breast Cancer with Humanized 2C4

The effect of rhuMAb 2C4 or HERCEPTIN® on human breast cancer cells which do not overexpress ErbB2 was assessed in a 3 day Alamar Blue assay (Ahmed, S. A. *J. Immunol. Methods* 170:211-224 (1994); and Page et al. *Int. J. Oncol.* 3:473-476 (1994)). The cells used in this assay were MDA-175 human breast cancer cells which express ErbB2 at a 1+ level. As shown in FIG. **12**, the growth of the breast cancer cell line, MDA-175, is significantly inhibited in a dose-dependent manner by the addition of rhuMAb 2C4 in comparison to HERCEPTIN® treatment.

The efficacy of rhuMAb 2C4 against MCF7 xenografts which are estrogen receptor positive (ER+) and express low levels of ErbB2 was assessed. Female mice supplemented with estrogen were used. rhuMAb 2C4 was administered at a dose of 30 mg/kg every week. As shown in FIG. **13**, rhuMAb 2C4 was effective in inhibiting breast cancer tumor growth in vivo, where the breast cancer was not characterized by overexpression of ErbB2.

EXAMPLE 8

Pharmacokinetics, Metabolism and Toxicology of 2C4

RhuMAb 2C4 was stable in human serum. No evidence or aggregates of complex formation in biological matrices was observed. In mice, rhuMAb 2C4 cleared faster than HERCEPTIN®. Pharmacokinetic studies indicate that weekly administration of about 2-6 mg/kg of rhuMAb 2C4 should result in serum concentrations similar to HERCEPTIN® as presently dosed. Resulting serum 2C4 exposure should greatly exceed IC_{50} determined in vitro.

A toxicology study was carried out in cynomolgus monkeys (2 males and 2 females per group). rhuMab 2C4 was administered intravenously at 0, 10, 50 or 100 mg/kg twice a week for 4 weeks. The toxicology study measurements included body weights (−2, −1 weeks and weekly thereafter); food consumption (qualitative, daily); physical examinations with assessment of blood pressure, electrocardiogram (ECG), and body temperature (−2, −1 weeks and weeks 2 and 4, 4 hours post-dose following that weeks second dose); cardiac ultrasound evaluations (following first dose week 1 and end of study, week 4); clinical pathology (baseline and end of weeks 2 and 4); urinalysis (baseline and end of weeks 2 and 4); antibody analysis sampling (baseline and end of weeks 2 and 4); as well as necropsy and histopathology analysis.

All animals in all groups survived to the end of the study. No significant clinical observations, or differences among groups, were noted. Necropsy results showed no significant gross abnormalities in organs from any animals. No significant microscopic abnormalities were observed by in tissues from any of the animals. No significant changes in ECG were noted from initiation to completion of the study. In addition, no differences among the groups were seen.

EXAMPLE 9

Dose Escalation

Cancer patients are administered a first dose of rhuMAb 2C4 at one of five dose levels (0.05, 0.5, 2.0, 4.0 or 10 mg/kg; 6 subjects per dose level), followed by a 4 week wash-out. Week 5 patients are given the same dose weekly 4 times followed by a further 4 week wash-out. Patients with complete response, partial response or stable disease are eligible for extension studies.

EXAMPLE 10

Therapy of Relapsed or Refractory Metastatic Prostate Cancer

RhuMAb 2C4 is a full-length, humanized monoclonal antibody (produced in CHO cells) directed against ErbB2. RhuMab 2C4 blocks the associated of ErbB2 with other ErbB family members thereby inhibiting intracellular signaling through the ErbB pathway. In contrast to HERCEPTIN®, rhuMAb 2C4 not only inhibits the growth of ErbB2 overexpressing tumors but also blocks growth of tumors that require ErbB ligand-dependent signaling.

RhuMAb 2C4 is indicated as a single agent for treatment of hormone-refractory (androgen independent) prostate cancer patients. Primary endpoints for efficacy include overall survival compared to best available care (Mitoxantrone/Prednisone), when used as a single agent, and safety. Secondary efficacy endpoints include: time to disease progression, response rate, quality of life, pain and/or duration of response. RhuMAb 2C4 is administered intravenously (IV) weekly or every three weeks at 2 or 4 mg/kg, respectively, until disease progression. The antibody is supplied as a multi-dose liquid formulation (20 mL fill at a concentration of 20 mg/mL or higher concentration).

RhuMAb 2C4 is also indicated in combination with chemotherapy for treatment of hormone-refractory (androgen independent) prostate cancer patients. Primary endpoints for efficacy include overall survival compared to chemotherapy, and safety. Secondary efficacy endpoints include: time to disease progression, response rate, quality of life, pain and/or duration of response. RhuMAb 2C4 is administered intravenously (IV) weekly or every three weeks at 2 or 4 mg/kg, respectively, until disease progression. The antibody is supplied as a multi-dose liquid formulation (20 mL fill at a concentration of 20 mg/mL or higher concentration).

Examples of drugs that can be combined with the anti-ErbB2 antibody (which blocks ligand activation of an ErbB2 receptor) to treat prostate cancer (e.g. androgen independent prostate cancer) include a farnesyl transferase inhibitor; an anti-angiogenic agent (e.g. an anti-VEGF antibody); an EGFR-targeted drug (e.g. C225 or ZD1839); another anti-ErbB2 antibody (e.g. a growth inhibitory anti-ErbB2 antibody such as HERCEPTIN®, or an anti-ErbB2 antibody which induces apoptosis such as 7C2 or 7F3, including humanized and/or affinity matured variants thereof); a cytokine (e.g. IL-2, IL-12, G-CSF or GM-CSF); an anti-androgen (such as flutamide or cyproterone acetate); leuprolide; suramin; a chemotherapeutic agent such as vinblastine, estra-

mustine, mitoxantrone, liarozole (a retinoic acid metabolism-blocking agent), cyclophosphamide, anthracycline antibiotics such as doxorubicin, a taxane (e.g. paclitaxel or docetaxel), or methotrexate, or any combination of the above, such as vinblastine/estramustine or cyclophosphamide/doxorubicin/methotrexate; prednisone; hydrocortizone; or combinations thereof. Standard doses for these various drugs can be administered, e.g. 40 mg/m^2/wk docetaxel (TAXOTERE®); 6 (AUC) carboplatin; and 200 mg/m^2 paclitaxel (TAXOL®).

EXAMPLE 11

Therapy of Metastatic Breast Cancer

RhuMAb 2C4 is indicated as a single agent for treatment of metastatic breast cancer patients whose tumors do not overexpress ErbB2. Primary endpoints for efficacy include response rate and safety. Secondary efficacy endpoints include: overall survival, time to disease progression, quality of life, and/or duration of response. RhuMAb 2C4 is administered intravenously (IV) weekly or every three weeks at 2 or 4 mg/kg, respectively, until disease progression. The antibody is supplied as a multi-dose liquid formulation (20 mL fill at a concentration of 20 mg/mL or higher concentration).

RhuMAb 2C4 is also indicated in combination with chemotherapy for treatment of metastatic breast cancer patients whose tumors do not overexpress ErbB2. Primary endpoints for efficacy include overall survival compared to chemotherapy alone, and safety. Secondary efficacy endpoints include: time to disease progression, response rate, quality of life, and/or duration of response. RhuMAb 2C4 is administered intravenously (IV) weekly or every three weeks at 2 or 4 mg/kg, respectively, until disease progression. The antibody is supplied as a multi-dose liquid formulation (20 mL fill at a concentration of 20 mg/mL or higher concentration).

Examples of drugs that can be combined with the anti-ErbB2 antibody (which blocks ligand activation of an ErbB2 receptor) to treat breast cancer (e.g. metastatic breast cancer which is not characterized by ErbB2 overexpression) include chemotherapeutic agents such as anthracycline antibiotics (e.g. doxorubicin), cyclophosphomide, a taxane (e.g. paclitaxel or docetaxel), navelbine, xeloda, mitomycin C, a platinum compound, oxaliplatin, gemcitabine, or combinations of two or more of these such as doxorubicin/cyclophosphomide; another anti-ErbB2 antibody (e.g. a growth inhibitory anti-ErbB2 antibody such as HERCEPTIN®, or an anti-ErbB2 antibody which induces apoptosis such as 7C2 or 7F3, including humanized or affinity matured variants thereof); an anti-estrogen (e.g. tamoxifen); a farnesyl transferase inhibitor; an anti-angiogenic agent (e.g. an anti-VEGF antibody); an EGFR-targeted drug (e.g. C225 or ZD1839); a cytokine (e.g. IL-2, IL-12, G-CSF or GM-CSF); or combinations of the above. Standard dosages for such additional drugs may be used.

RhuMAb 2C4 is additionally indicated in combination with HERCEPTIN® for treatment of metastatic breast cancer patients whose tumors overexpress ErbB2. Primary endpoints for efficacy include response rate, and safety. Secondary efficacy endpoints include: time to disease progression, overall survival compared to HERCEPTIN® alone, quality of life, and/or duration of response. RhuMAb 2C4 is administered intravenously (IV) weekly or every three weeks at 2 or 4 mg/kg, respectively, until disease progression. The antibody is supplied as a multi-dose liquid formulation (20 mL fill at a concentration of 20 mg/mL or higher concentration). HERCEPTIN® is administered IV as an initial loading dose of 4 mg/kg followed by a weekly maintenance dose of 2 mg/kg. HERCEPTIN® is supplied as a lyophilized powder. Each vial of HERCEPTIN® contains 440 mg HERCEPTIN®, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 400 mg α-α-trehalose dihydrate, and 1.8 mg polysorbate 20. Reconstitution with 20 mL of Bacteriostatic Water for Injection (BWFI) containing 1.1% benzyl alcohol as a preservative, yields 21 mL of a multi-dose solution containing 21 mg/mL HERCEPTIN®, at a pH of approximately 6.0.

EXAMPLE 12

Therapy of Lung Cancer

RhuMAb 2C4 is indicated as a single agent for treatment of stage IIIb or IV non-small cell lung cancer (NSCLC). Primary endpoints for efficacy include response rate, and safety. Secondary efficacy endpoints include: overall survival, time to disease progression, quality of life, and/or duration of response. RhuMAb 2C4 is administered intravenously (IV) weekly or every three weeks at 2 or 4 mg/kg, respectively, until disease progression. The antibody is supplied as a multi-dose liquid formulation (20 mL fill at a concentration of 20 mg/mL or higher concentration).

RhuMAb 2C4 is also indicated in combination with chemotherapy for treatment of metastatic non-small cell lung cancer patients. Primary endpoints for efficacy include overall survival compared to standard therapy, and safety. Secondary efficacy endpoints include: time to disease progression, response rate, quality of life and/or duration of response. RhuMAb 2C4 is administered intravenously (IV) weekly or every three weeks at 2 or 4 mg/kg, respectively, until disease progression. The antibody is supplied as a multi-dose liquid formulation (20 mL fill at a concentration of 20 mg/mL or higher concentration).

Examples of additional drugs which can be combined with the antibody (which binds ErbB2 and blocks ligand activation of an ErbB receptor) to treat lung cancer, include chemotherapeutic agents such as carboplatin, a taxane (e.g. paclitaxel or docetaxel), gemcitabine, navelbine, cisplatin, oxaliplatin, or combinations of any of these such as carboplatin/docetaxel; another anti-ErbB2 antibody (e.g. a growth inhibitory anti-ErbB2 antibody such as HERCEPTIN®, or an anti-ErbB2 antibody which induces apoptosis such as 7C2 or 7F3, including humanized or affinity matured variants thereof); a farnesyl transferase inhibitor; an anti-angiogenic agent (e.g. an anti-VEGF antibody); an EGFR-targeted drug (e.g. C225 or ZD1839); a cytokine (e.g. IL-2, IL-12, G-CSF or GM-CSF); or combinations of the above.

EXAMPLE 13

Therapy of Colorectal Cancer

RhuMAb 2C4 is indicated as a single agent for treatment of metastatic colorectal cancer. Primary endpoints for efficacy include response rate and safety. Secondary efficacy endpoints include: overall survival, time to disease progression, quality of life, and/or duration of response. RhuMAb 2C4 is administered intravenously (IV) weekly or every three weeks at 2 or 4 mg/kg, respectively, until disease progression. The antibody is supplied as a multi-dose liquid formulation (20 mL fill at a concentration of 20 mg/mL or higher concentration).

RhuMAb 2C4 is also indicated in combination with chemotherapy for treatment of metastatic colorectal cancer patients. Primary endpoints for efficacy include overall survival compared to standard therapy, and safety. Secondary

efficacy endpoints include: time to disease progression, response rate, quality of life, and/or duration of response. RhuMAb 2C4 is administered intravenously (IV) weekly or every three weeks at 2 or 4 mg/kg, respectively, until disease progression. The antibody is supplied as a multi-dose liquid formulation (20 mL fill at a concentration of 20 mg/mL or higher concentration).

Examples of chemotherapeutic agents used to treat colorectal cancer, which can be combined with the antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor, include 5-fluorouracil (5-FU), leucovorin (LV), CPT-11, levamisole, or combinations of any two or more of these, e.g., 5-FU/LV/CPT-11. Standard dosages of such chemotherapeutic agents can be administered. Other drugs that may be combined with the anti-ErbB2 antibody to treat colorectal cancer include a farnesyl transferase inhibitor; an anti-angiogenic agent (e.g. an anti-VEGF antibody); an EGFR-targeted drug (e.g. C225 or ZD1839); a cytokine (e.g. IL-2, IL-12, G-CSF or GM-CSF); another anti-ErbB2 antibody (e.g. a growth inhibitory anti-ErbB2 antibody such as HERCEPTIN®, or an anti-ErbB2 antibody which induces apoptosis such as 7C2 or 7F3, including humanized or affinity matured variants thereof); or combinations of the above.

SEQUENCE LISTING

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35 40 45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Asp
50 55 60

Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
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Ile Lys

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35 40 45

Glu Trp Ile Gly Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
50 55 60

Asn Gln Arg Phe Lys Gly Lys Ala Ser Leu Thr Val Asp Arg Ser
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Ser Arg Ile Val Tyr Met Glu Leu Arg Ser Leu Thr Phe Glu Asp
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Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
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Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
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Ile Lys


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Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
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35 40 45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
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Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys

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20 25 30

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35 40 45

Glu Trp Val Ala Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
50 55 60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
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Lys Gly

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<210> SEQ ID NO 13
<211> LENGTH: 645
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<213> ORGANISM: Homo sapiens

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Leu Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp
20 25 30

Met Lys Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met
35 40 45

Leu Arg His Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu
50 55 60

Glu Leu Thr Tyr Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln
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                 80                  85                  90

Val Arg Gln Val Pro Leu Gln Arg Leu Arg Ile Val Arg Gly Thr
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Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala Val Leu Asp Asn Gly
                110                 115                 120

Asp Pro Leu Asn Asn Thr Thr Pro Val Thr Gly Ala Ser Pro Gly
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                140                 145                 150

Gly Gly Val Leu Ile Gln Arg Asn Pro Gln Leu Cys Tyr Gln Asp
                155                 160                 165

Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn Asn Gln Leu Ala
                170                 175                 180

Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys His Pro Cys
                185                 190                 195

Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser Ser Glu
                200                 205                 210

Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys Ala
                215                 220                 225

Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys
                230                 235                 240

Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys
                245                 250                 255

Leu His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala
                260                 265                 270

Leu Val Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro
                275                 280                 285

Glu Gly Arg Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro
                290                 295                 300

Tyr Asn Tyr Leu Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys
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Cys Glu Lys Cys Ser Lys Pro Cys Ala Arg Val Cys Tyr Gly Leu
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Gly Met Glu His Leu Arg Glu Val Arg Ala Val Thr Ser Ala Asn
                350                 355                 360

Ile Gln Glu Phe Ala Gly Cys Lys Lys Ile Phe Gly Ser Leu Ala
                365                 370                 375

Phe Leu Pro Glu Ser Phe Asp Gly Asp Pro Ala Ser Asn Thr Ala
                380                 385                 390

Pro Leu Gln Pro Glu Gln Leu Gln Val Phe Glu Thr Leu Glu Glu
                395                 400                 405

Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro Asp Ser Leu Pro
                410                 415                 420

Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg Gly Arg Ile
                425                 430                 435

Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu Gly Ile
                440                 445                 450

Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly Leu
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Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val
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Cys His Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro
                515                 520                 525

Thr Gln Cys Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys
                530                 535                 540

Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val
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Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
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Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val
                575                 580                 585

Ala Cys Ala His Tyr Lys Asp Pro Pro Phe Cys Val Ala Arg Cys
                590                 595                 600

Pro Ser Gly Val Lys Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys
                605                 610                 615

Phe Pro Asp Glu Glu Gly Ala Cys Gln Pro Cys Pro Ile Asn Cys
                620                 625                 630

Thr His Ser Cys Val Asp Leu Asp Asp Lys Gly Cys Pro Ala Glu
                635                 640                 645
```

What is claimed is:

1. A method of treating ErbB2-expressing breast cancer in a human, comprising administering to the human therapeutically effective amounts of (a) huMAb4D5-8 antibody; and (b) humanized 2C4 antibody variant 574.

2. The method of claim **1** wherein the huMAb4D5-8 antibody and the humanized 2C4 antibody are coadministered, using separate formulations or a single formulation.

3. The method of claim l wherein the huMAb4D5-8 antibody and the humanized 2C4 antibody are consecutively administered in either order.

4. The method of claim **3** wherein there is a time period during which both the huMAb4D5-8 antibody and the humanized 2C4 antibody simultaneously exert their biological activities.

5. A method of treating ErbB2-expressing breast cancer in a human, comprising administering to the human therapeutically effective amounts of (a) huMAb4D5-8 antibody; and (b) humanized 2C4 antibody comprising the variable light (V_L) domain amino acid sequence in SEQ ID NO:3, and the variable heavy (V_H) domain amino acid sequence in SEQ ID NO:4.

6. The method of claim **5** wherein the huMAb4D5-8 antibody and the humanized 2C4 antibody are coadministered, using separate formulations or a single formulation.

7. The method of claim **5** wherein the huMAb4D5-8 antibody and the humanized 2C4 antibody are consecutively administered in either order.

8. The method of claim **7** wherein there is a time period during which both the huMAb4D5-8 antibody and the humanized 2C4 antibody simultaneously exert their biological activities.

9. The method of claim **5** wherein the humanized 2C4 antibody is an intact IgG1 antibody.

* * * * *

EXHIBIT M

US007449184B2

(12) **United States Patent**
Allison et al.

(10) **Patent No.: US 7,449,184 B2**
(45) **Date of Patent: Nov. 11, 2008**

(54) **FIXED DOSING OF HER ANTIBODIES**

(75) Inventors: **David E. Allison**, San Mateo, CA (US); **Rene Bruno**, Marseilles (FR); **Jian-Feng Lu**, Foster City, CA (US); **Chee M. Ng**, San Mateo, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 121 days.

(21) Appl. No.: **11/154,091**

(22) Filed: **Jun. 15, 2005**

(65) **Prior Publication Data**

US 2006/0165702 A1 Jul. 27, 2006

Related U.S. Application Data

(60) Provisional application No. 60/645,697, filed on Jan. 21, 2005.

(51) **Int. Cl.**
A61K 39/365 (2006.01)
C07K 16/30 (2006.01)

(52) **U.S. Cl.** **424/138.1**; 424/130.1; 424/141.1; 424/143.1; 530/387.1; 530/388.1; 530/388.8; 530/388.85; 514/2

(58) **Field of Classification Search** ..... None
See application file for complete search history.

(56) **References Cited**

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(57) **ABSTRACT**

The present invention concerns fixed dosing of HER antibodies, such as Pertuzumab.

32 Claims, 18 Drawing Sheets

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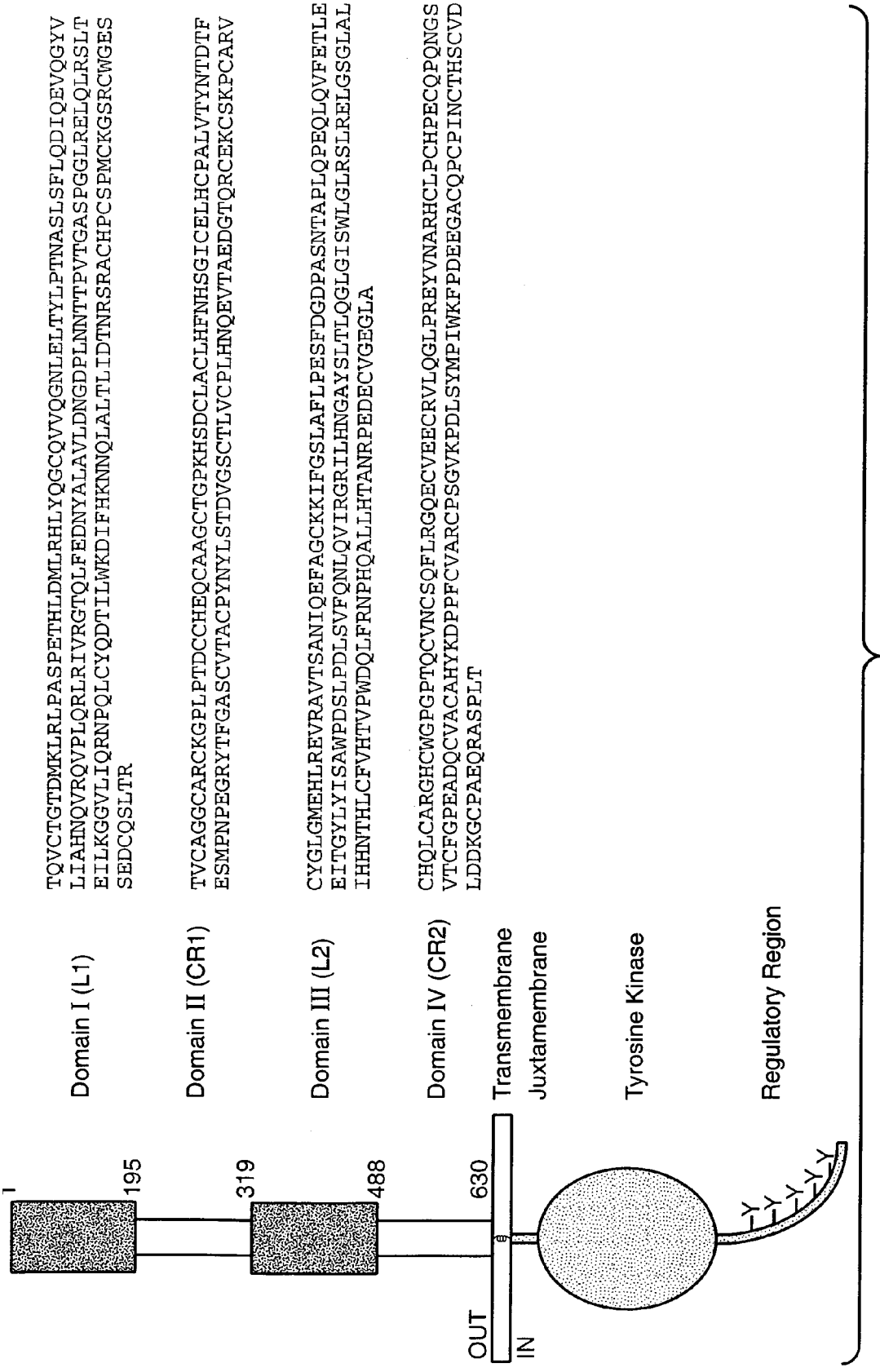
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EILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGES
SEDCQSLTR
TVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTF
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CYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLE
EITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLAL
IHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLA
CHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGS
VTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVD
LDDKGCPAEQRASPLT
1
195
319
488
630
OUT
IN
Domain I (L1)
Domain II (CR1)
Domain III (L2)
Domain IV (CR2)
Transmembrane
Juxtamembrane
Tyrosine Kinase
Regulatory Region
Y
Y
Y
Y
Y

FIG._1

VARIABLE LIGHT

```
                10        20          30          40
2C4       DTVMTQSHKIMSTSVGDRVSITC [KASQDVSIGVA] WYQQRP
           **    **** *      *                    *
574       DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA] WYQQKP
                                   *   ** ***
hum κI    DIQMTQSPSSLSASVGDRVTITC [RASQSISNYLA] WYQQKP


                50          60        70        80
2C4       GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA
           **                  *  *         *    * *
574       GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISSLQP
                     * *****
hum κI    GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP


                90         100
2C4       EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:1)
            * *                  *   *
574       EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:3)
                       *** *
hum κI    EDFATYYC [QQYNSLPWT] FGQGTKVEIK (SEQ ID NO:5)
```

FIG._2A

VARIABLE HEAVY

```
                10        20          30          40
2C4       EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS
              **  **  *  * ***  *                  * *
574       EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA
                                      ** * *
hum III   EVQLVESGGGLVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA


                50  a      60          70        80
2C4       HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM
          *  *   **                     *** *    **** *
574       PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL
                     ****** *** ****       * * *
hum III   PGKGLEWVA [VISGDGGSTYYADSVKG] RFTISRDNSKNTLYL


            abc      90         100ab         110
2C4       ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLTVSS (SEQ ID NO:2)
          ***  **                          **
574       QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTLVTVSS (SEQ ID NO:4)
                            ********
hum III   QMNSLRAEDTAVYYCAR [GRVGYSLYDY] WGQGTLVTVSS (SEQ ID NO:6)
```

FIG._2B

Amino Acid Sequence for Pertuzumab Light Chain

```
1       10        20        30        40        50        60
|       |         |         |         |         |         |
DIQMTQSPSSLSASVGDRVTITC**KASQDVSIGVA**WYQQKPGKAPKLLIYS**ASYRYT**GVPS

        70        80        90        100       110       120
        |         |         |         |         |         |
RFSGSGSGTDFTLTISSLQPEDFATYYC**QQYYIYPYT**FGQGTKVEIKRTVAAPSVFIFPP

        130       140       150       160       170       180
        |         |         |         |         |         |
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT

        190       200       210
        |         |         |
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
```

FIG._3A

Amino Acid Sequence for Pertuzumab Heavy Chain

```
1       10        20        30        40        50        60
|       |         |         |         |         |         |
EVQLVESGGGLVQPGGSLRLSCAAS**GFTFTDYTMD**WVRQAPGKGLEWVA**DVNPNSGGSIY**

        70        80        90        100       110       120
        |         |         |         |         |         |
**NQRFK**GRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARN**LGPSFYFDY**WGQGTLVTVSSA

        130       140       150       160       170       180
        |         |         |         |         |         |
STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG

        190       200       210       220       230       240
        |         |         |         |         |         |
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGP

        250       260       270       280       290       300
        |         |         |         |         |         |*
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS

        310       320       330       340       350       360
        |         |         |         |         |         |
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM

        370       380       390       400       410       420
        |         |         |         |         |         |
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ

        430       440       448
        |         |         |
QGNVFSCSVMHEALHNHYTQKSLSLSPG
```

FIG._3B

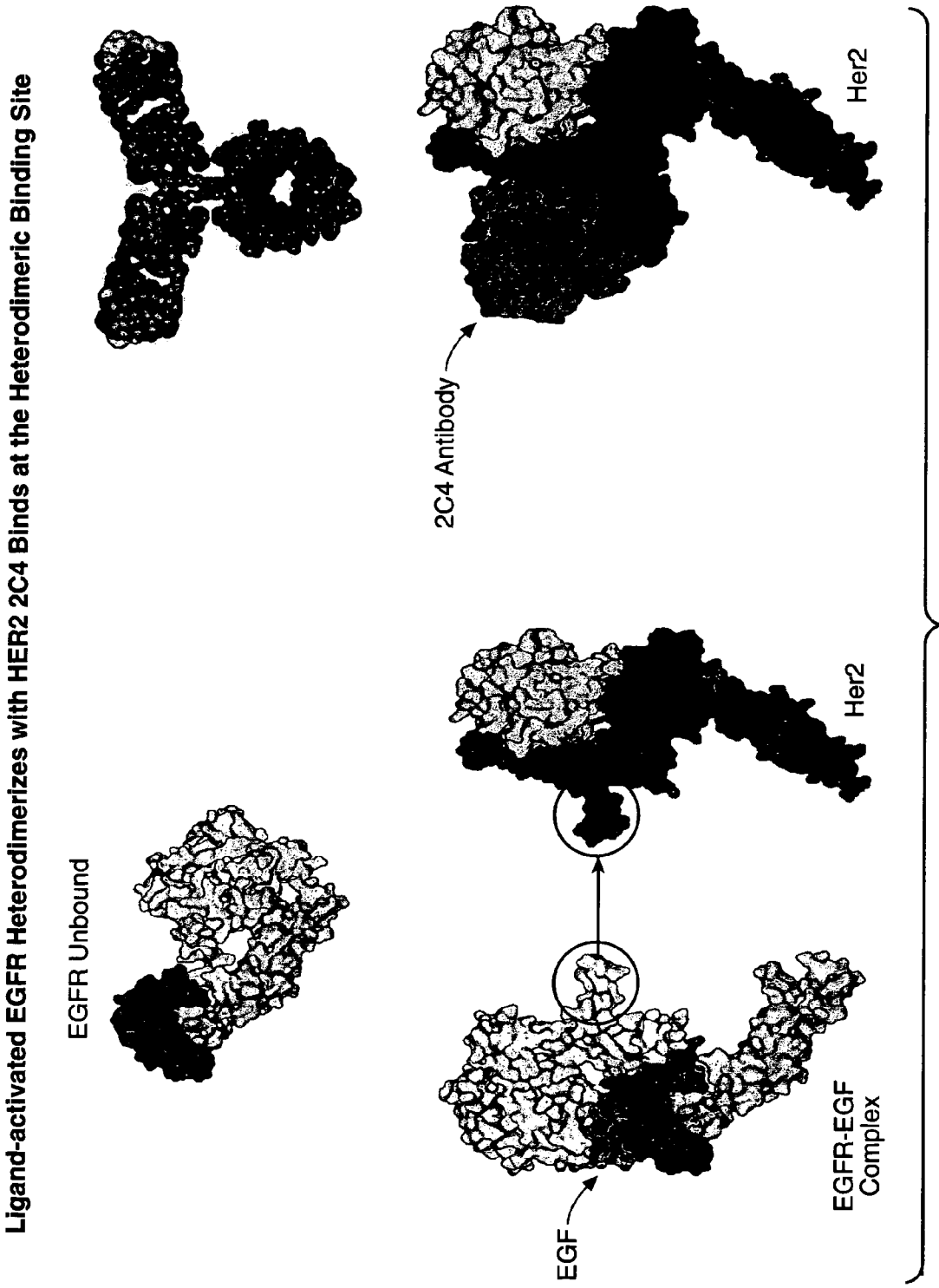

Ligand-activated EGFR Heterodimerizes with HER2 2C4 Binds at the Heterodimeric Binding Site
EGFR Unbound
2C4 Antibody
Her2
Her2
EGF
EGFR-EGF Complex

FIG._4

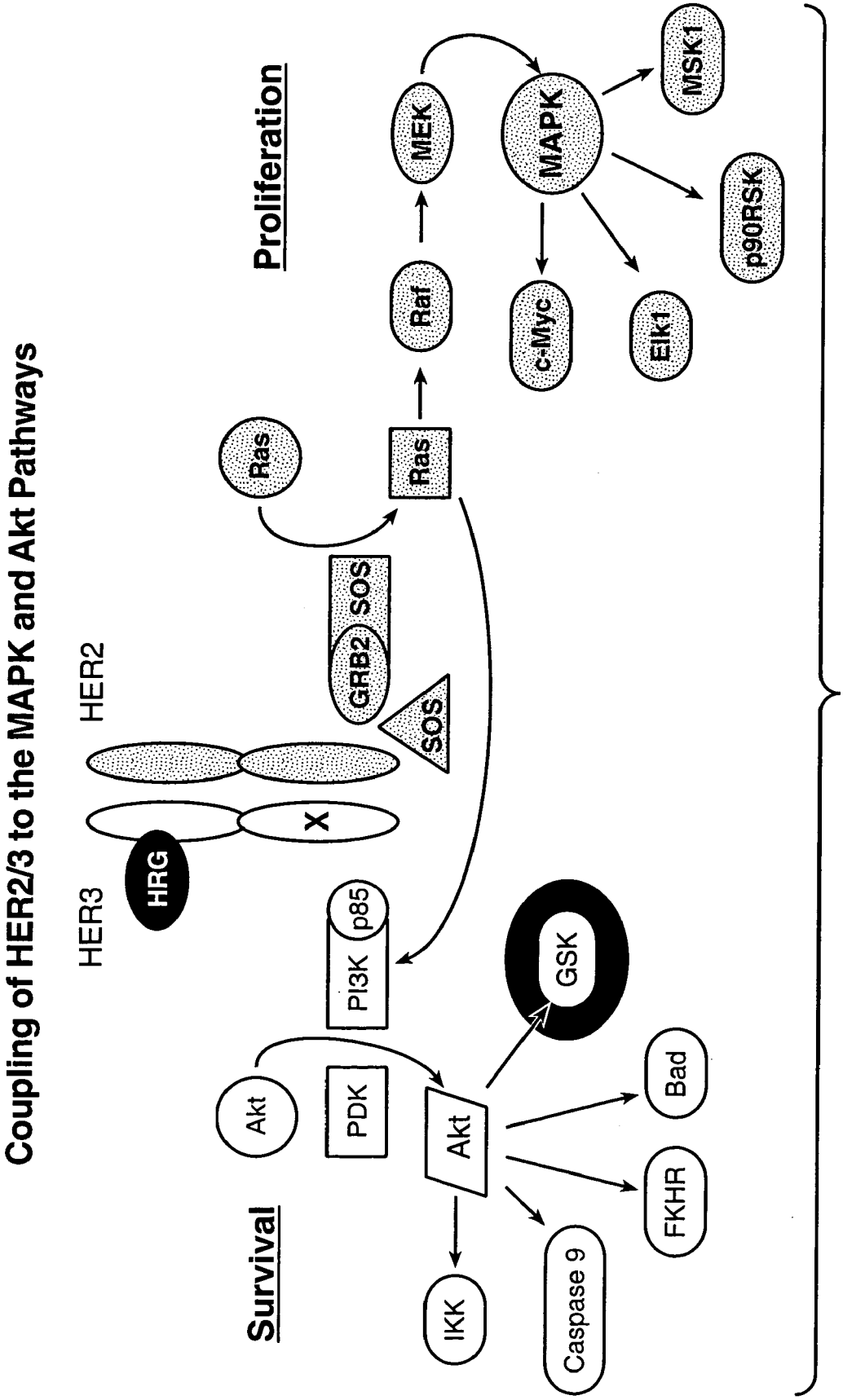
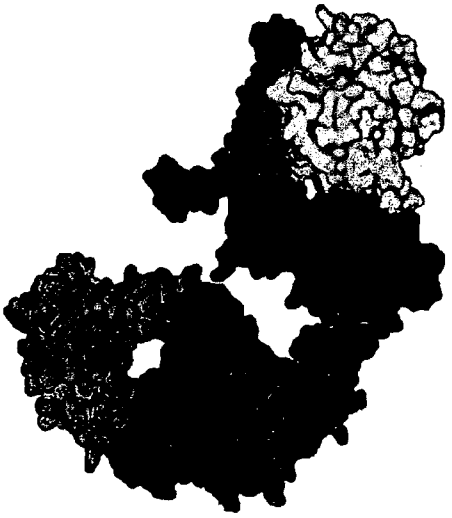

Coupling of HER2/3 to the MAPK and Akt Pathways
HER3
HER2
HRG
X
Ras
GRB2
SOS
SOS
Ras
Raf
MEK
MAPK
MSK1
p90RSK
c-Myc
Elk1
Proliferation
PI3K
p85
GSK
Akt
PDK
Akt
Bad
FKHR
IKK
Caspase 9
Survival

FIG._5

Trastuzumab
Herceptin



- Binds in IV near JM.
- Protects against receptor shedding
- Moderately affects receptor down-modulation
- Slight effect on HER2's role as a coreceptor

Pertuzumab
Omnitarg

- Binds in II at dimerization interface
- Does not prevent receptor shedding
- Moderately affects receptor down-modulation
- Major effect on HER2's role as a coreceptor

FIG._6

LIGHT CHAIN

```
1   D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q D V N T A V A W Y Q Q K P G K A P K
46  L L I Y S A S F L Y S G V P S R F S G S R S G T D F T L T I S S L Q P E D F A T Y Y C Q Q
91  H Y T T P P T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L
136 L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T
181 L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
```

FIG._7A

HEAVY CHAIN

```
1   E V Q L V E S G G G L V Q P G G S L R L S C A A S G F N I K D T Y I H W V R Q A P G K G L
46  E W V A R I Y P T N G Y T R Y A D S V K G R F T I S A D T S K N T A Y L Q M N S L R A E D
91  T A V Y Y C S R W G G D G F Y A M D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S
136 K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S
181 G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K
226 T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S
271 H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D
316 W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E
361 M T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G
406 S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
```

FIG._7B

```
1                                           15                                            30                                            45
V H S D I Q M T Q S P S S L S A S V G D R V T I T C K A S Q D V S I G V A W Y Q Q K P G K
46                                          60                                            75                                            90
A P K L L I Y S A S Y R Y T G V P S R F S G S G S G T D F T L T I S S L Q P E D F A T Y Y
91                                          105                                           120                                           135
C Q Q Y Y I Y P Y T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V
136                                         150                                           165                                           180
V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S
181                                         195                                           210                   217
T L T L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
```

FIG._8A

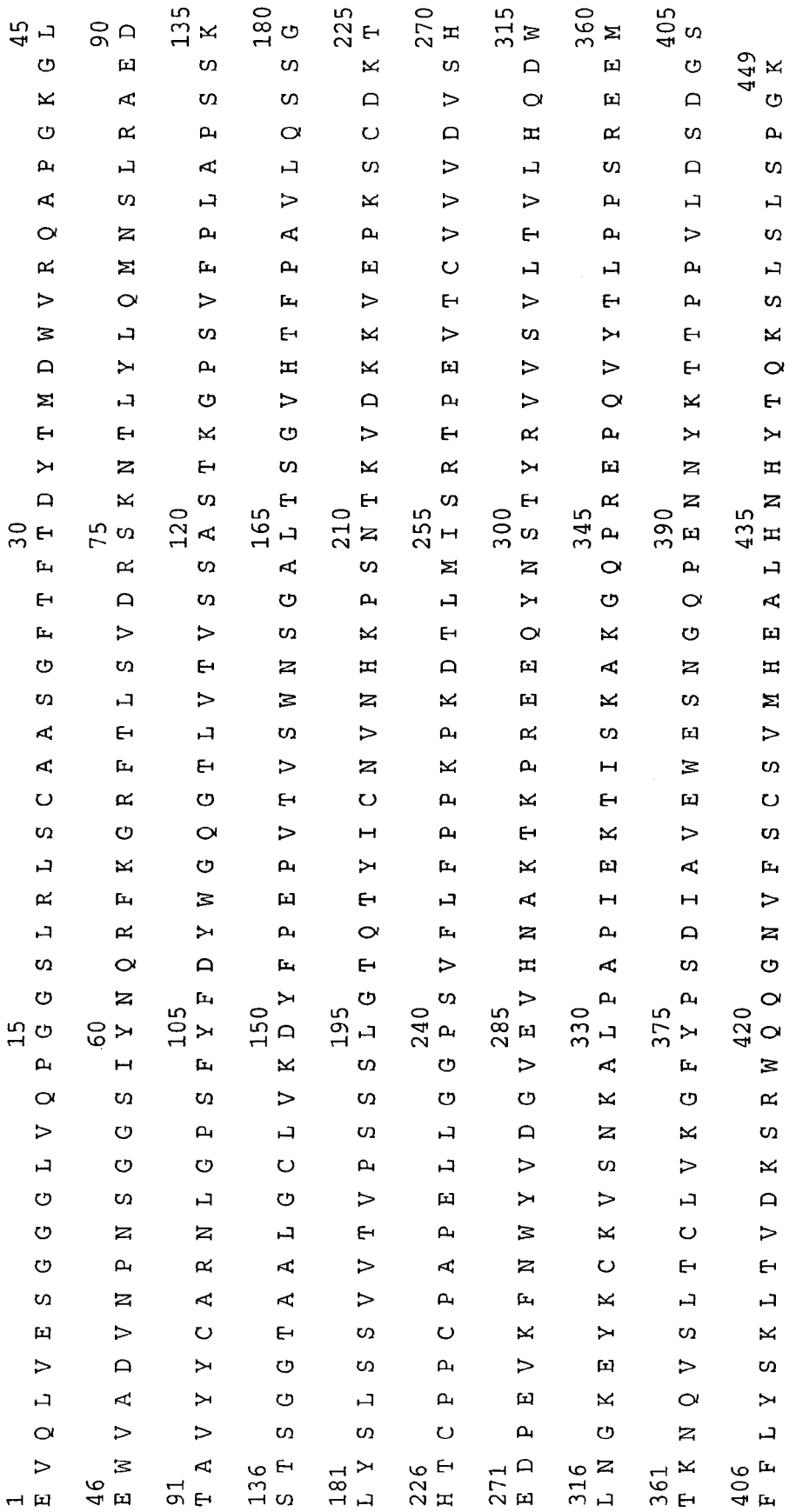

1 E V Q L V E S G G G L V Q P G G S L R L S C A A S G F T F T D Y T M D W V R Q A P G K G L
46 E W V A D V N P N S G G S I Y N Q R F K G R F T L S V D R S K N T L Y L Q M N S L R A E D
91 T A V Y Y C A R N L G P S F Y F D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S K
136 S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G
181 L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K T
226 H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H
271 E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W
316 L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E M
361 T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S
406 F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
15
30
45
60
75
90
105
120
135
150
165
180
195
210
225
240
255
270
285
300
315
330
345
360
375
390
405
420
435
449

FIG._8B

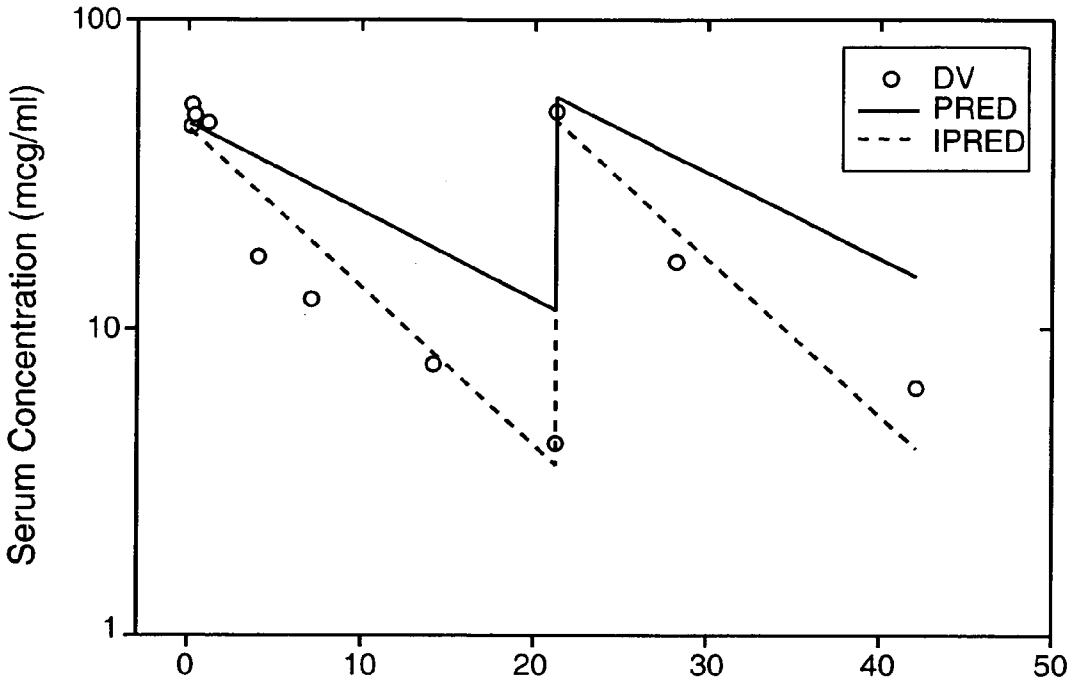

100
10
1
Serum Concentration (mcg/ml)
0
10
20
30
40
50
Time (Days)
DV
PRED
IPRED

FIG._9A

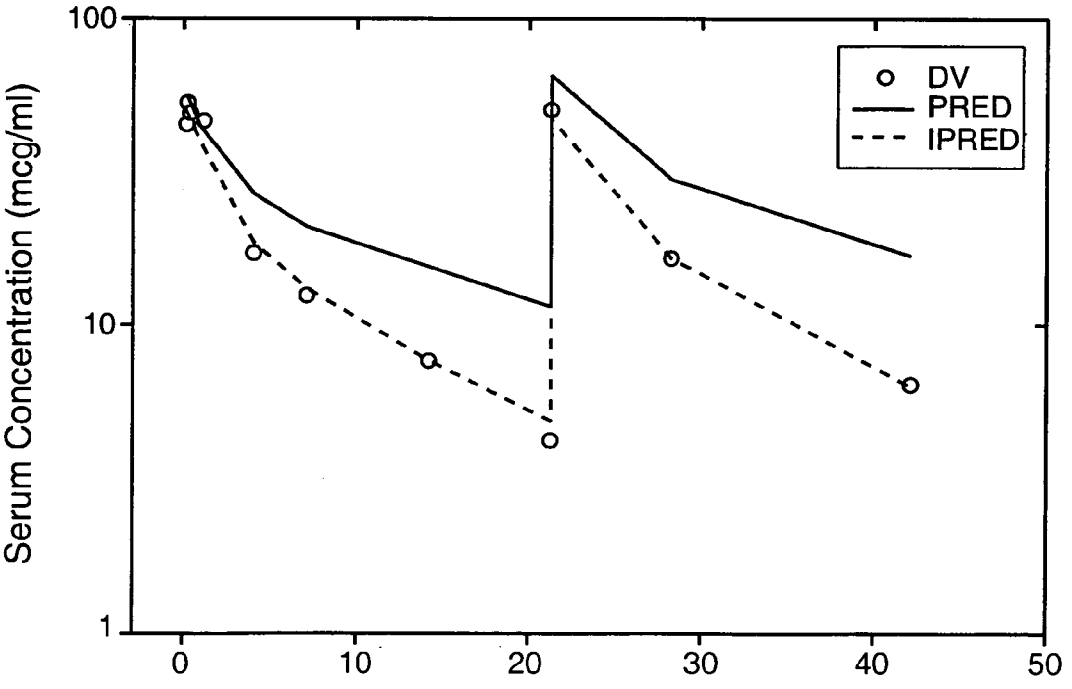

100
10
1
Serum Concentration (mcg/ml)
0
10
20
30
40
50
Time (Days)
DV
PRED
IPRED

FIG._9B

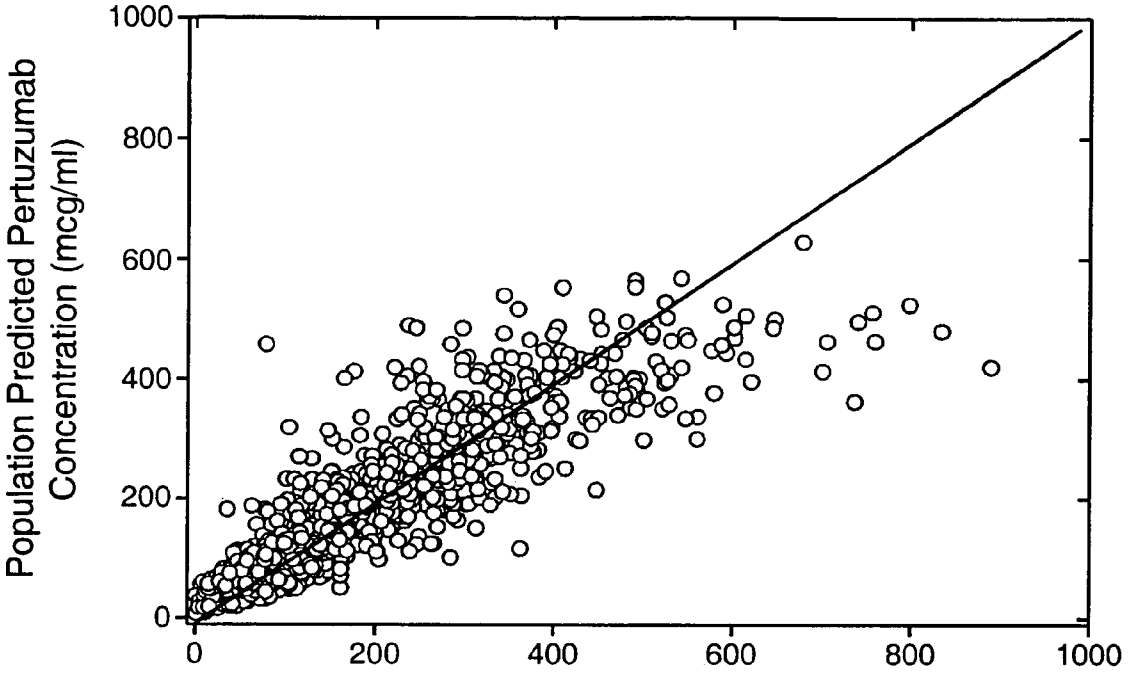


FIG._10A

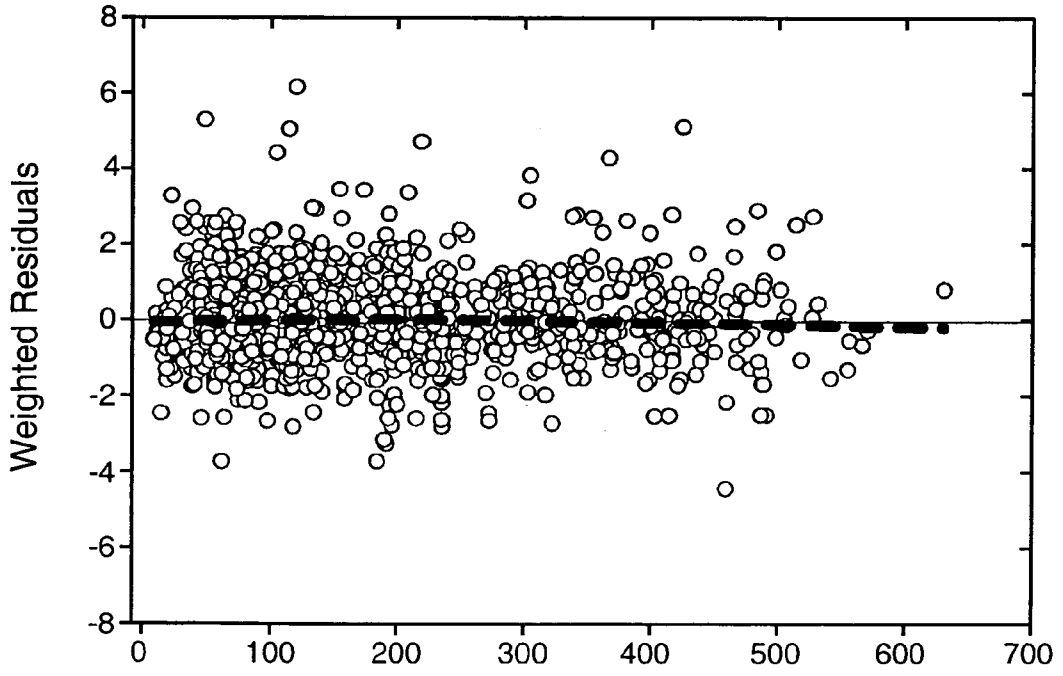
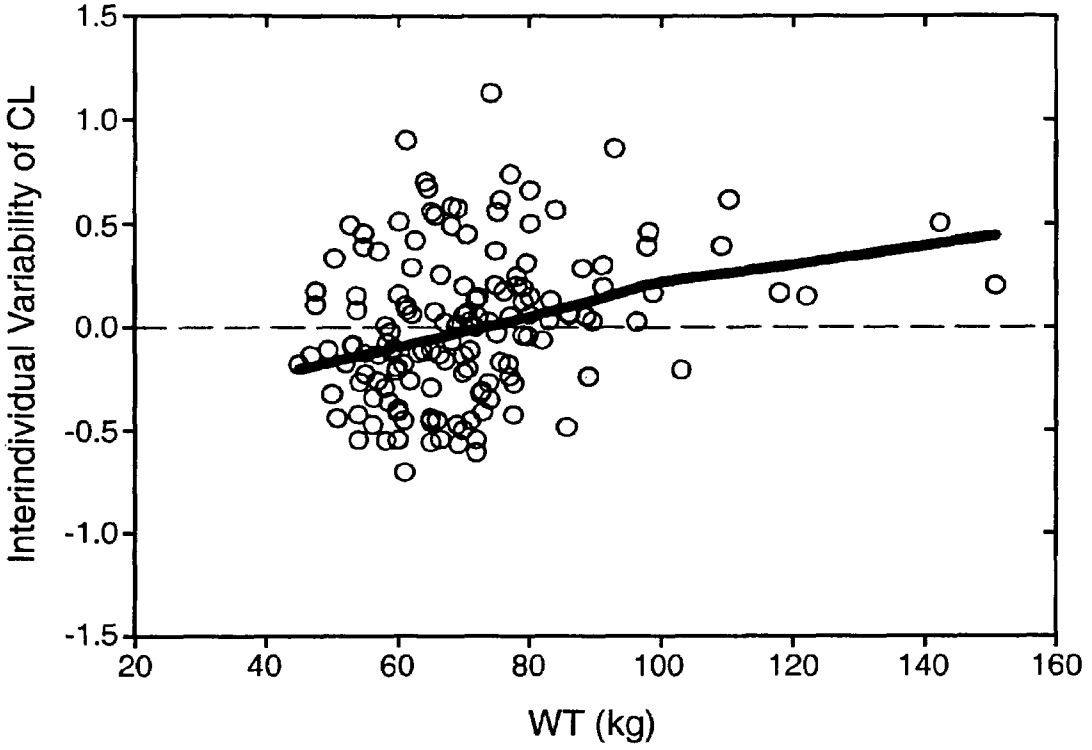


FIG._10B


Interindividual Variability of CL
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
20
40
60
80
100
120
140
160
WT (kg)

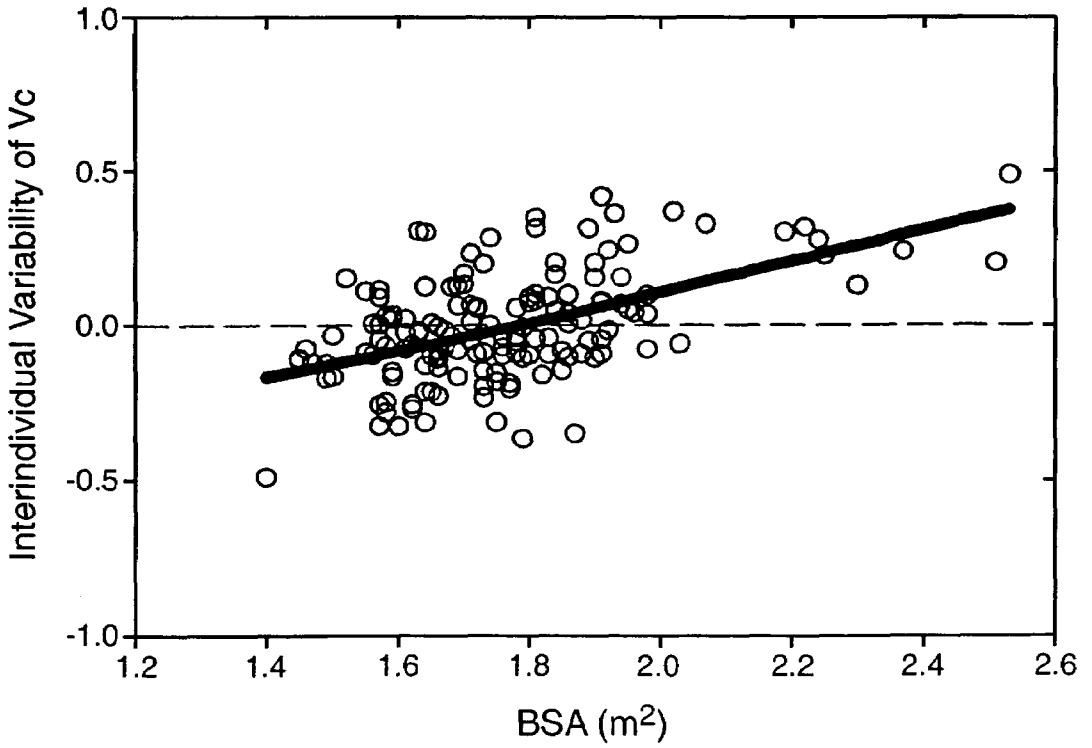
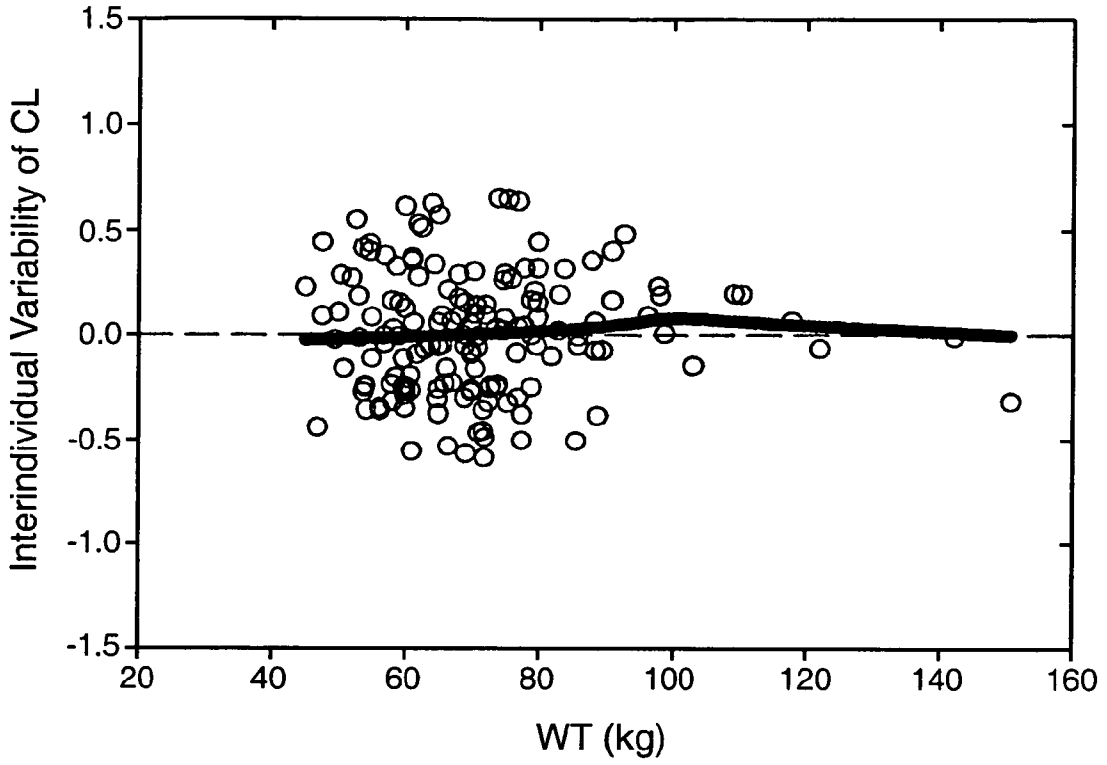

Interindividual Variability of Vc
1.0
0.5
0.0
-0.5
-1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
BSA (m2)

FIG._11A


Interindividual Variability of CL
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
20
40
60
80
100
120
140
160
WT (kg)

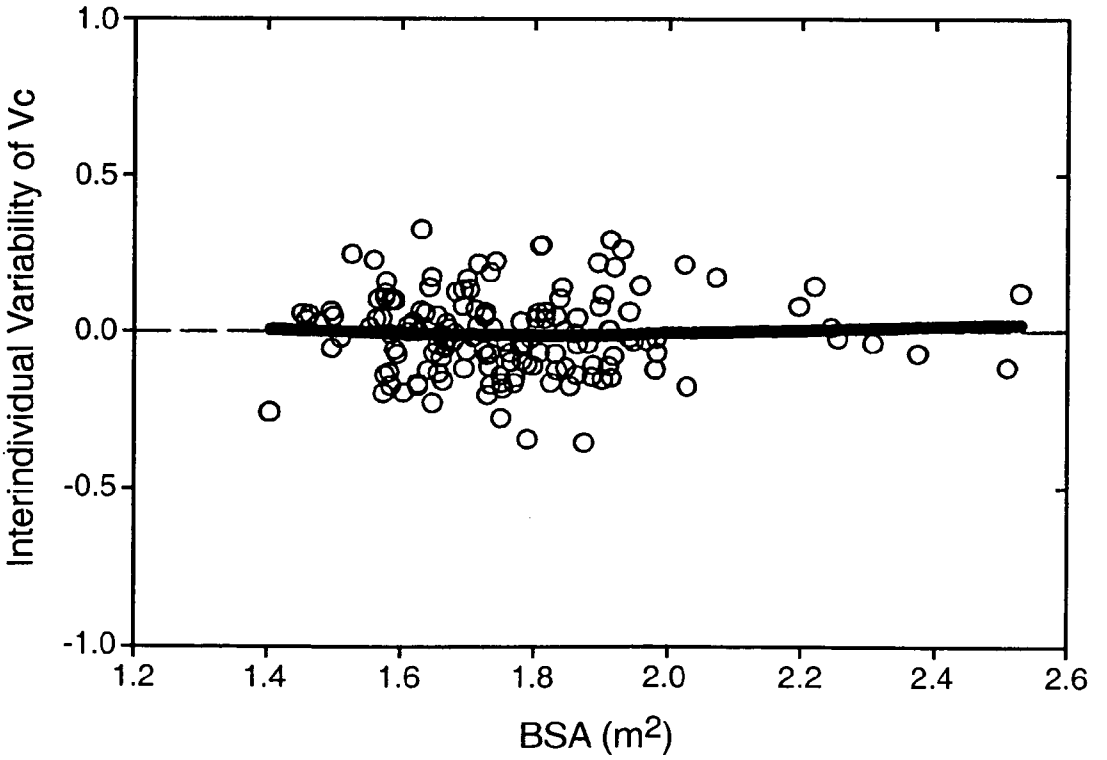

Interindividual Variability of Vc
1.0
0.5
0.0
-0.5
-1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
BSA (m2)

FIG._11B

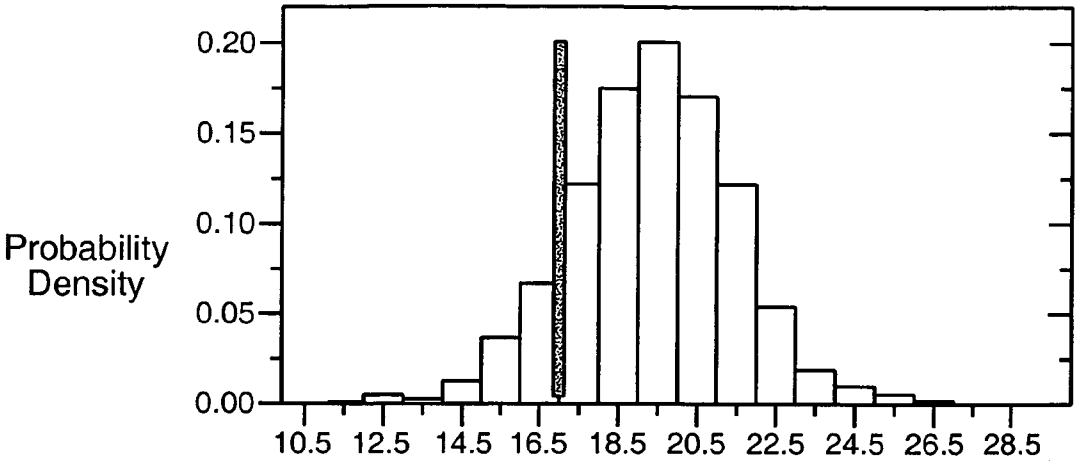


FIG._12A

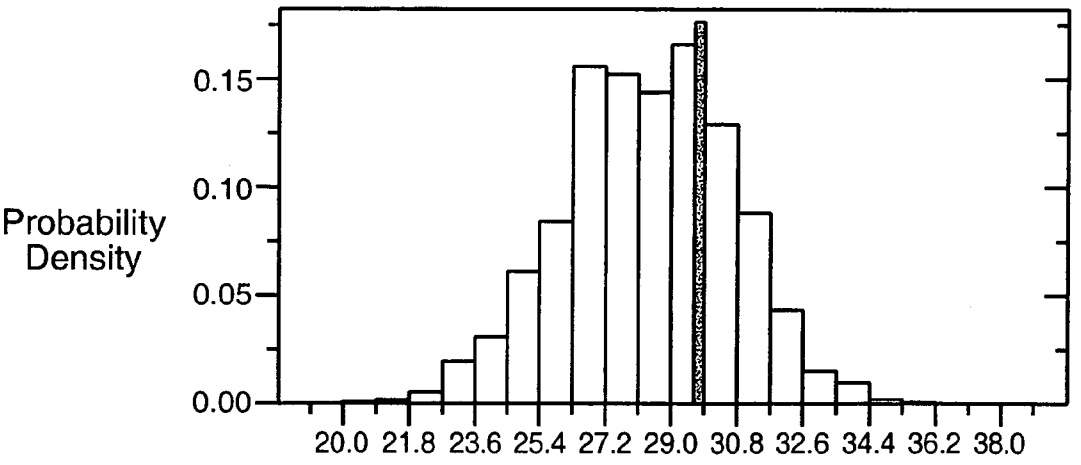


FIG._12B

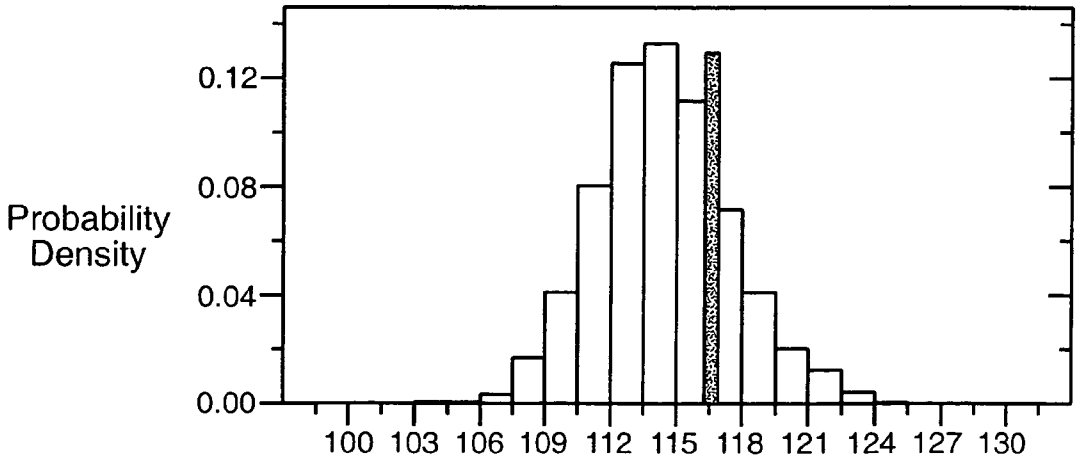


FIG._12C

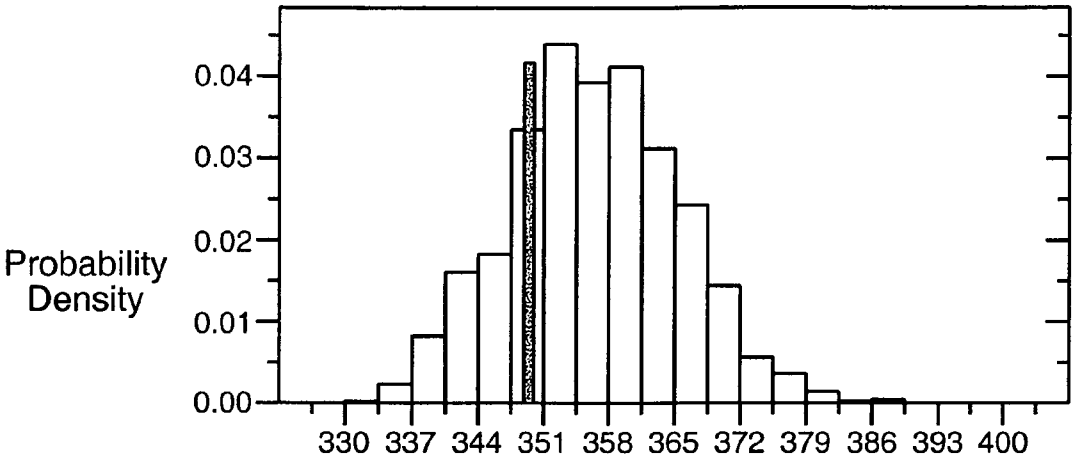


FIG._12D

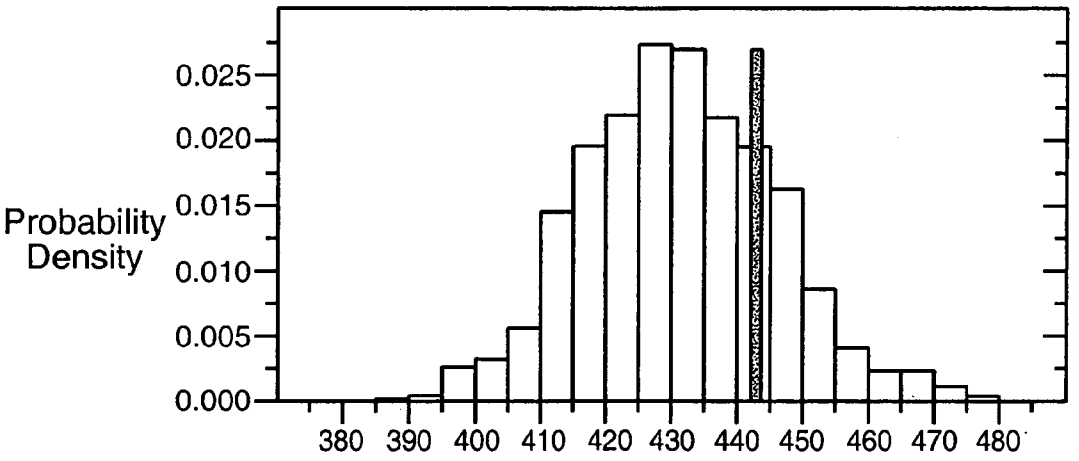


FIG._12E

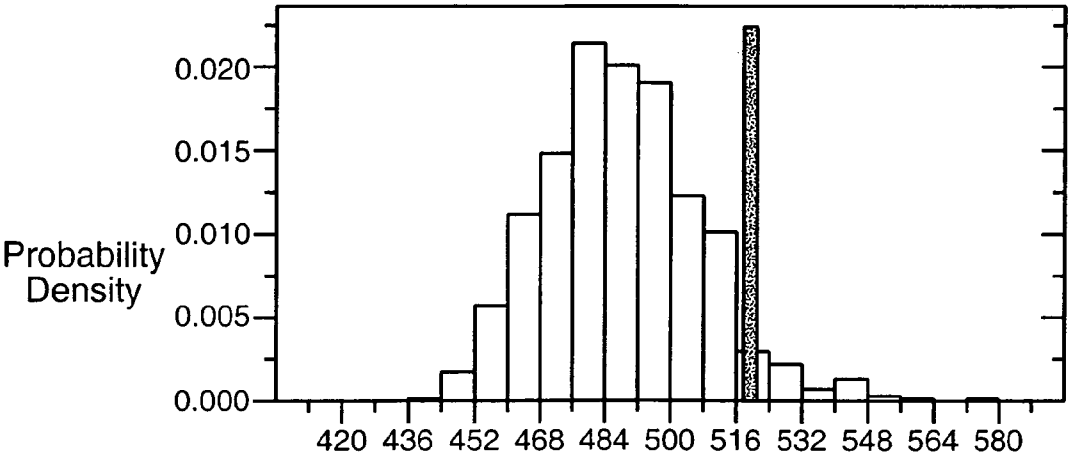


FIG._12F

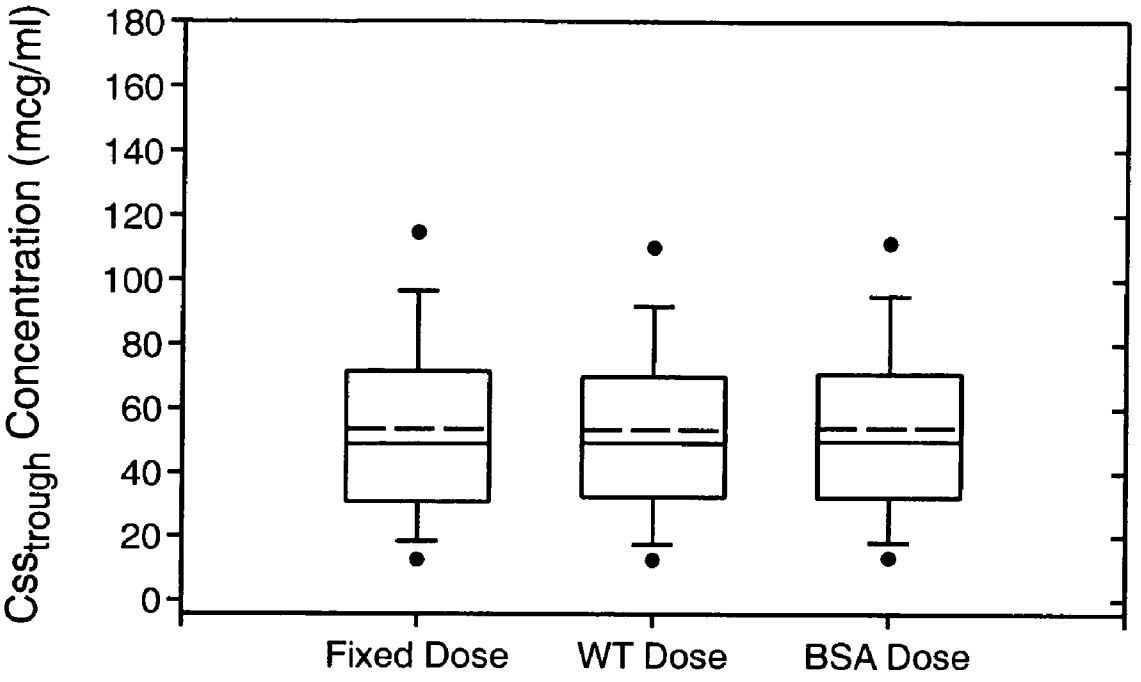

Css_{trough} Concentration (mcg/ml)
180
160
140
120
100
80
60
40
20
0
Fixed Dose
WT Dose
BSA Dose

FIG._13

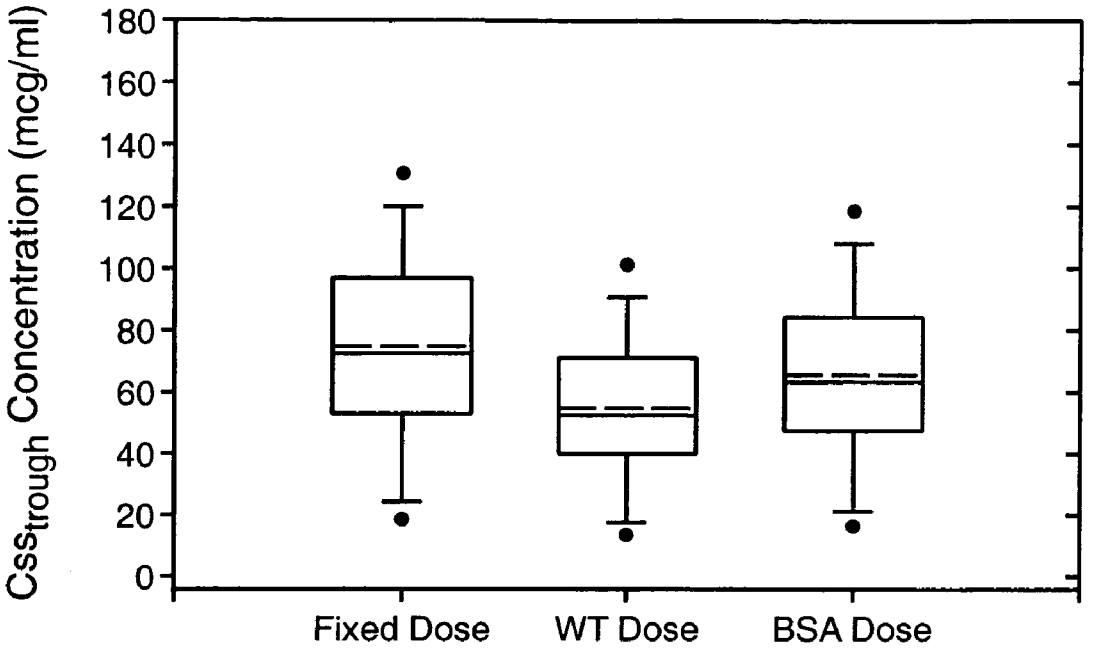


FIG._14A

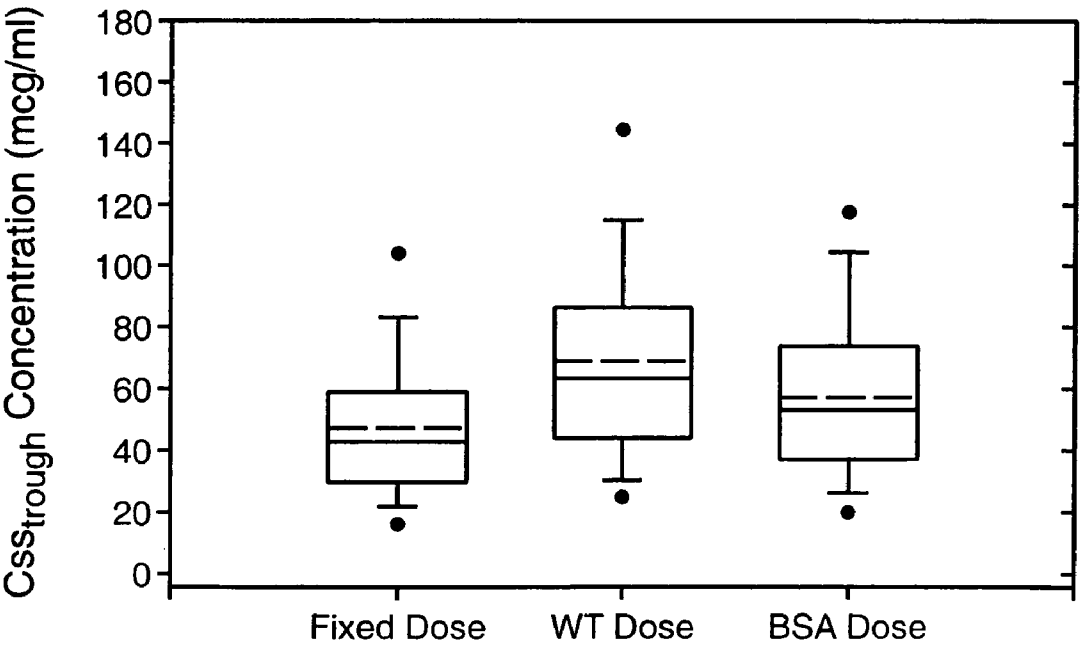


FIG._14B

FIXED DOSING OF HER ANTIBODIES

This is a non-provisional application filed under 37 CFR 1.53(b) claiming priority to provisional application 60/645,697 filed Jan. 21, 2005, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns fixed dosing of HER antibodies, such as pertuzumab.

BACKGROUND OF THE INVENTION

HER Receptors and Antibodies Thereagainst

The HER family of receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes four distinct members including epidermal growth factor receptor (EGFR, ErbB1, or HER1), HER2 (ErbB2 or p185^{neu}), HER3 (ErbB3) and HER4 (ErbB4 or tyro2).

EGFR, encoded by the erbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, transforming growth factor alpha (TGF-α), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn, *Pharmac. Ther.* 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-α and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn, supra; Masui et al. *Cancer Research* 44:1002-1007 (1984); and Wu et al. *J. Clin. Invest.* 95:1897-1905 (1995).

The second member of the HER family, p185^{neu}, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., *Science,* 235:177-182 (1987); Slamon et al., *Science,* 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. See, among others, King et al., *Science,* 229:974 (1985); Yokota et al., *Lancet:* 1:765-767 (1986); Fukushige et al., *Mol Cell Biol.,* 6:955-958 (1986); Guerin et al., *Oncogene Res.,* 3:21-31 (1988); Cohen et al., *Oncogene,* 4:81-88 (1989); Yonemura et al., *Cancer Res.,* 51:1034 (1991); Borst et al., *Gynecol. Oncol.,* 38:364 (1990); Weiner et al., *Cancer Res.,* 50:421-425 (1990); Kern et al., *Cancer Res.,* 50:5184 (1990); Park et al., *Cancer Res.,* 49:6605 (1989); Zhau et al., *Mol. Carcinog.,* 3:254-257 (1990); Aasland et al. *Br. J. Cancer* 57:358-363 (1988); Williams et al., Pathobiology 59:46-52 (1991); and McCann et al., *Cancer,* 65:88-92 (1990). HER2 may be overexpressed in prostate cancer (Gu et al., *Cancer Lett.* 99:185-9 (1996); Ross et al., *Hum. Pathol.* 28:827-33 (1997); Ross et al., *Cancer* 79:2162-70 (1997); and Sadasivan et al., *J. Urol.* 150:126-31 (1993)).

Antibodies directed against the rat p185^{neu} and human HER2 protein products have been described.

Drebin and colleagues have raised antibodies against the rat neu gene product, p185^{neu} See, for example, Drebin et al., *Cell* 41:695-706 (1985); Myers et al., *Meth. Enzym.* 198:277-290 (1991); and WO94/22478. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions of p185^{neu} result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. See also U.S. Pat. No. 5,824,311 issued Oct. 20, 1998.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of HER2 antibodies which were characterized using the human breast tumor cell line SK-BR-3. Relative cell proliferation of the SK-BR-3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also U.S. Pat. No. 5,677,171 issued Oct. 14, 1997. The HER2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11 (3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 1(2):979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis et al. *Cancer Research* 56:1457-1465 (1996); and Schaefer et al. *Oncogene* 15:1385-1394 (1997).

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein.

Other HER2 antibodies with various properties have been described in Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991); McKenzie et al. *Oncogene* 4:543-548 (1989); Maier et al. *Cancer Res.* 51:5361-5369 (1991); Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al. *PNAS* (USA) 88:8691-8695 (1991); Bacus et al. *Cancer Research* 52:2580-2589 (1992); Xu et al. *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992); Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al. *Oncogene* 14:2099-2109 (1997).

Homology screening has resulted in the identification of two other HER receptor family members; HER3 (U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al., *PNAS* (USA) 86:9193-9197 (1989)) and HER4 (EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-

475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The HER receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of HER ligands (Earp et al. *Breast Cancer Research and Treatment* 35: 115-132 (1995)). EGFR is bound by six different ligands; epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin, heparin binding epidermal growth factor (HB-EGF), betacellulin and epiregulin (Groenen et al. *Growth Factors* 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta and gamma heregulins (Holmes et al., *Science,* 256:1205-1210 (1992); U.S. Pat. No. 5,641,869; and Schaefer et al. *Oncogene* 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motor neuron derived factor (SMDF). For a review, see Groenen et al. *Growth Factors* 11:235-257 (1994); Lemke, G. *Molec. & Cell. Neurosci.* 7:247-262 (1996) and Lee et al. *Pharm. Rev.* 47:51-85 (1995). Recently three additional HER ligands were identified; neuregulin-2 (NRG-2) which is reported to bind either HER3 or HER4 (Chang et al. *Nature* 387 509-512 (1997); and Carraway et al *Nature* 387:512-516 (1997)); neuregulin-3 which binds HER4 (Zhang et al. *PNAS* (USA) 94(18):9562-7 (1997)); and neuregulin-4 which binds HER4 (Harari et al. *Oncogene* 18:2681-89 (1999)) HB-EGF, betacellulin and epiregulin also bind to HER4.

While EGF and TGFα do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase. See Earp et al., supra. Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski et al., *J. Biol. Chem.,* 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi et al., *Journal of Neuroscience* 15: 1329-1340(1995); Morrissey et al., *Proc. Natl. Acad. Sci. USA* 92: 1431-1435 (1995); and Lewis et al., *Cancer Res.,* 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, *Cell* 78:5-8 (1994)).

Patent publications related to HER antibodies include: U.S. Pat. No. 5,677,171, U.S. Pat. No. 5,720,937, U.S. Pat. No. 5,720,954, U.S. Pat. No. 5,725,856, U.S. Pat. No. 5,770,195, U.S. Pat. No. 5,772,997, U.S. Pat. No. 6,165,464, U.S. Pat. No. 6,387,371, U.S. Pat. No. 6,399,063, US2002/0192211A1, U.S. Pat. No. 6,015,567, U.S. Pat. No. 6,333,169, U.S. Pat. No. 4,968,603, U.S. Pat. No. 5,821,337, U.S. Pat. No. 6,054,297, U.S. Pat. No. 6,407,213, U.S. Pat. No. 6,719,971, U.S. Pat. No. 6,800,738, US2004/0236078A1, U.S. Pat. No. 5,648,237, U.S. Pat. No. 6,267,958, U.S. Pat. No. 6,685,940, U.S. Pat. No. 6,821,515, WO98/17797, U.S. Pat. No. 6,127,526, U.S. Pat. No. 6,333,398, U.S. Pat. No. 6,797,814, U.S. Pat. No. 6,339,142, U.S. Pat. No. 6,417,335, U.S. Pat. No. 6,489,447, WO99/31140, US2003/0147884A1, US2003/0170234A1, US2005/0002928A1, U.S. Pat. No. 6,573,043, US2003/0152987A1, WO99/48527, US2002/0141993A1, WO01/00245, US2003/0086924, US2004/0013667A1, WO00/69460, WO01/00238, WO01/15730, U.S. Pat. No. 6,627,196B 1, U.S. Pat. No. 6,632,979B 1, WO01/00244, US2002/0090662A1, WO01/89566, US2002/0064785, US2003/0134344, WO 04/24866, US2004/0082047, US2003/0175845A1, WO03/087131, US2003/0228663, WO2004/008099A2, US2004/0106161, WO2004/048525, US2004/0258685A1, U.S. Pat. No. 5,985,553, U.S. Pat. No. 5,747,261, U.S. Pat. No. 4,935,341, U.S. Pat. No. 5,401,638, U.S. Pat. No. 5,604,107, WO 87/07646, WO 89/10412, WO 91/05264, EP 412,116 B1, EP 494,135 B1, U.S. Pat. No. 5,824,311, EP 444,181 B1, EP 1,006,194 A2, US 2002/0155527A1, WO 91/02062, U.S. Pat. No. 5,571,894, U.S. Pat. No. 5,939,531, EP 502,812 B1, WO 93/03741, EP 554,441 B1, EP 656,367 A1, U.S. Pat. No. 5,288,477, U.S. Pat. No. 5,514,554, U.S. Pat. No. 5,587,458, WO 93/12220, WO 93/16185, U.S. Pat. No. 5,877,305, WO 93/21319, WO 93/21232, U.S. Pat. No. 5,856,089, WO 94/22478, U.S. Pat. No. 5,910,486, U.S. Pat. No. 6,028,059, WO 96/07321, U.S. Pat. No. 5,804,396, U.S. Pat. No. 5,846,749, EP 711,565, WO 96/16673, U.S. Pat. No. 5,783,404, U.S. Pat. No. 5,977,322, U.S. Pat. No. 6,512,097, WO 97/00271, U.S. Pat. No. 6,270,765, U.S. Pat. No. 6,395,272, U.S. Pat. No. 5,837,243, WO 96/40789, U.S. Pat. No. 5,783,186, U.S. Pat. No. 6,458,356, WO 97/20858, WO 97/38731, U.S. Pat. No. 6,214,388, U.S. Pat. No. 5,925,519, WO 98/02463, U.S. Pat. No. 5,922,845, WO 98/18489, WO 98/33914, U.S. Pat. No. 5,994,071, WO 98/45479, U.S. Pat. No. 6,358,682 B1, US 2003/0059790, WO 99/55367, WO 01/20033, US 2002/0076695 A1, WO 00/78347, WO 01/09187, WO 01/21192, WO 01/32155, WO 01/53354, WO 01/56604, WO 01/76630, WO02/05791, WO 02/11677, U.S. Pat. No. 6,582,919, US2002/0192652A1, US 2003/0211530A1, WO 02/44413, US 2002/0142328, U.S. Pat. No. 6,602,670 B2, WO 02/45653, WO 02/055106, US 2003/0152572, US 2003/0165840, WO 02/087619, WO 03/006509, WO03/012072, WO 03/028638, US 2003/0068318, WO 03/041736, EP 1,357,132, US 2003/0202973, US 2004/0138160, U.S. Pat. No. 5,705,157, U.S. Pat. No. 6,123,939, EP 616,812 B1, US 2003/0103973, US 2003/0108545, U.S. Pat. No. 6,403,630 B1, WO 00/61145, WO 00/61185, U.S. Pat. No. 6,333,348 B1, WO 01/05425, WO 01/64246, US 2003/0022918, US 2002/0051785 A1, U.S. Pat. No. 6,767,541, WO 01/76586, US 2003/0144252, WO 01/87336, US 2002/0031515 A1, WO 01/87334, WO 02/05791, WO 02/09754, US 2003/0157097, US 2002/0076408, WO 02/055106, WO 02/070008, WO 02/089842 and WO 03/86467.

Diagnostics

Patients treated with the HER2 antibody trastuzumab are selected for therapy based on HER2 overexpression/amplification. See, for example, WO99/31140 (Paton et al.), US2003/0170234A1 (Hellmann, S.), and US2003/0147884 (Paton et al.); as well as WO01/89566, US2002/0064785, and US2003/0134344 (Mass et al.). See, also, US2003/0152987, Cohen et al., concerning immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) for detecting HER2 overexpression and amplification.

WO2004/053497 (Bacus et al.) refers to determining or predicting response to HERCEPTIN® therapy. US2004/013297A1 (Bacus et al.) concerns determining or predicting response to ABX0303 EGFR antibody therapy. WO2004/000094 (Bacus et al.) is directed to determining response to GW572016, a small molecule, EGFR-HER2 tyrosine kinase inhibitor. WO2004/063709, Amler et al., refers to biomarkers and methods for determining sensitivity to EGFR inhibitor, erlotinib HCl. US2004/0209290, Cobleigh et al., concerns gene expression markers for breast cancer prognosis.

Patients treated with pertuzumab can be selected for therapy based on HER activation or dimerization. Patent pub-

lications concerning pertuzumab and selection of patients for therapy therewith include: WO01/00245 (Adams et al.); US2003/0086924 (Sliwkowski, M.); US2004/0013667A1 (Sliwkowski, M.); as well as WO2004/008099A2, and US2004/0106161 (Bossenmaier et al.).

Cronin et al., *Am. J. Path.* 164(1): 35-42 (2004) describes measurement of gene expression in archival paraffin-embedded tissues. Ma et al. *Cancer Cell* 5:607-616 (2004) describes gene profiling by gene oligonucleotide microarray using isolated RNA from tumor-tissue sections taken from archived primary biopsies.

Dosing of Anticancer Drugs and HER Antibodies

Papers discussing dosing of anticancer drugs include: Egorin, M. *J Clin Oncol* 2003; 21:182-3 (2003); Baker et al. *J Natl Cancer Inst* 94:1883-8 (2002); Felici et al. *Eur J Cancer* 38:1677-84 (2002); Loos et al. *Clin. Cancer Res.* 6:2685-9(2000); de Jongh et al. *J. Clin Oncol.* 19:3733-9 (2001); Mathijssen et al. *J. Clin Oncol.* 20:81-7 (2002); and de Jong et al. *Clin Cancer Res* 10:4068-71 (2004).

Typically, commercially available humanized IgG monoclonal antibodies (i.e. trastuzumab, and bevacizumab, Genentech Inc., South San Francisco and gemtuzumab ozogomicin, Wyeth Pharmaceuticals, Philadelphia) and cytotoxic small molecule drugs in oncology have been administered on a weight -based (mg/kg) or body surface area-based (BSA) dosing method.

Cetuximab (ERBITUX®) is an antibody that binds EGF receptor and is approved for therapy of colorectal cancer. In colorectal cancer, cetuximab 400 mg/m2 is given as a loading dose by intravenous infusion over 2 hours. This is followed by once weekly maintenance doses of 250 mg/m2 given over 1 hour. See cetuximab prescribing information.

Trastuzumab (HERCEPTIN®) is administered to patients with metastatic breast cancer as a 4 mg/kg loading dose, followed by weekly 2 mg/kg doses. See trastuzumab prescribing information.

See, also, WO99/31140; US2003/0147884A1; US2003/0170234A1; US2005/0002928A1; WO00/69460; WO01/15730 and U.S. Pat. No. 6,627,196B1 concerning trastuzumab dosing.

Pertuzumab (also known as recombinant human monoclonal antibody 2C4; OMNITARG™, Genentech, mc, South San Francisco) represents the first in a new class of agents known as HER dimerization inhibitors (HDI) and functions to inhibit the ability of HER2 to form active heterodimers with other HER receptors (such as EGFR/HER1, HER3 and HER4) and is active irrespective of HER2 expression levelsEN.REFLIST. See, for example, Harari and Yarden Oncogene 19:6102-14 (2000); Yarden and Sliwkowski. *Nat Rev Mol Cell Biol* 2:127-37 (2001); Sliwkowski *Nat Struct Biol* 10:158-9 (2003); Cho etal. *Nature* 421:756-60 (2003); and Malik et al. *Pro Am Soc Cancer Res* 44:176-7 (2003).

Pertuzumab blockade of the formation of HER2-HER3 heterodimers in tumor cells has been demonstrated to inhibit critical cell signaling, which results in reduced tumor proliferation and survival (Agus et al. *Cancer Cell* 2:127-37 (2002)).

Pertuzumab has undergone testing as a single agent in the clinic with a phase Ia trial in patients with advanced cancers and phase II trials in patients with ovarian cancer and breast cancer as well as lung and prostate cancer. In a Phase I study, patients with incurable, locally advanced, recurrent or metastatic solid tumors that had progressed during or after standard therapy were treated with pertuzumab given intravenously every 3 weeks. Pertuzumab was generally well tolerated. Tumor regression was achieved in 3 of 20 patients evaluable for response. Two patients had confirmed partial responses. Stable disease lasting for more than 2.5 months was observed in 6 of 21 patients (Agus et al. *Pro Am Soc Clin Oncol* 22:192 (2003)). At doses of 2.0-15 mg/kg, the pharmacokinetic of pertuzumab was linear, and mean clearance ranged from 2.69 to 3.74 mL/day/kg and the mean terminal elimination half-life ranged from 15.3 to 27.6 days. Antibodies to pertuzumab were not detected (Allison et al. *Pro Am Soc Clin Oncol* 22:197 (2003)). Pertuzumab was dosed on a weight-basis (mg/kg) in the Phase I trial. Phase II trials have been initiated using a fixed-dose.

SUMMARY OF THE INVENTION

The present invention provides the first critical assessment of the impact and utility of fixed dosing of a humanized IgG1 monoclonal antibody on pharmacokinetics and target drug concentrations. The primary objectives of this analysis of the HER antibody pertuzumab were to: 1) evaluate the population pharmacokinetic and predictive covariates for pertuzumab in cancer patients, and 2) examine the variability of steady-state trough concentrations and exposures after fixed, or body weight-, and body surface area (BSA)-based dosing.

Accordingly, in a first aspect, the invention provides a method for treating cancer comprising administering one or more fixed dose(s) of a HER antibody to a human patient in an amount effective to treat the cancer.

In another aspect, the invention provides a method of treating cancer in a human patient comprising administering at least one fixed dose of pertuzumab to the patient, wherein the fixed dose is selected from the group consisting of approximately 420 mg, approximately 525 mg, approximately 840 mg, and approximately 1050 mg of pertuzumab.

The invention also concerns an article of manufacture comprising a vial containing a fixed dose of a HER antibody, wherein the fixed dose is selected from the group consisting of approximately 420 mg, approximately 525 mg, approximately 840 mg, and approximately 1050 mg of the HER antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** provides a schematic of the HER2 protein structure, and amino acid sequences for Domains I-IV (SEQ ID Nos.19-22, respectively) of the extracellular domain thereof.

FIGS. **2**A and **2**B depict alignments of the amino acid sequences of the variable light (V_L) (FIG. **2**A) and variable heavy (V_H) (FIG. **2**B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 1 and 2, respectively); V_L and V_H domains of humanized 2C4 version 574 (SEQ ID Nos. 3 and 4, respectively), and human V_L and V_H consensus frameworks (hum κ1, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 5 and 6, respectively). Asterisks identify differences between humanized 2C4 version 574 and murine monoclonal antibody 2C4 or between humanized 2C4 version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets.

FIGS. **3**A and **3**B show the amino acid sequences of pertuzumab light chain and heavy chain (SEQ ID Nos. 13 and 14, respectively). CDRs are shown in bold. Calculated molecular mass of the light chain and heavy chain are 23,526.22 Da and 49,216.56 Da (cysteines in reduced form). The carbohydrate moiety is attached to Asn 299 of the heavy chain.

FIG. **4** depicts, schematically, binding of 2C4 at the heterodimeric binding site of HER2, thereby preventing het-

erodimerization with activated EGFR or HER3. FIG. **5** depicts coupling of HER2/HER3 to the MAPK and Akt pathways.

FIG. **6** compares various activities of trastuzumab and pertuzumab.

FIGS. **7**A and **7**B show the amino acid sequences of trastuzumab light chain (FIG. **7**A; SEQ ID No. 15) and heavy chain (FIG. **7**B; SEQ ID No. 16), respectively.

FIGS. **8**A and **8**B depict a variant pertuzumab light chain sequence (FIG. **8**A; SEQ ID No. 17) and a variant pertuzumab heavy chain sequence (FIG. **8**B; SEQ ID No. 18), respectively.

FIGS. **9**A and **9**B are representative profiles of a single subject's PK data fitted by a one-(FIG. **9**A) or two-(FIG. **9**B) compartmental model. Open circles indicate observed concentration. Solid and dotted lines indicate population predicted and individual predicted concentration, respectively.

FIGS. **10**A and **10**B represent model diagnostic plots. FIG. **10**A depicts observed versus predicted pertuzumab concentrations. The solid line is the line of unity. FIG. **10**B depicts weighted residuals versus predicted pertuzumab concentrations. The dashed line is a LOESS smooth of data.

FIGS. **11**A and **11**B depict random effect (η) for clearance (CL) by weight (WT) and volume at the central compartment (Vc) by body surface area (BSA) for the base model (FIG. **11**A) and final model (FIG. **11**B).

FIGS. **12**A-F show model evaluation of pertuzumab final population pharmacokinetic model using a posterior model check. Posterior predictive distribution and observed values for the test statistics: FIG. **12**A-2.5th; FIG. **12**B-5th; FIG. **12**C-50th, FIG. **12**D-90th, FIG. **12**E-95th, FIG. **12**F-97.5th. The vertical line on each histogram represents the observed value of the test statistic.

FIG. **13** illustrates the predicted pertuzumab steady state trough concentration (Day 84) after a fixed, weight (WT) or BSA-based dose for 1000 simulated subjects bootstrapped from original pharmacokinetic (PK) dataset according to the final model.

FIGS. **14**A and **14**B show the predicted pertuzumab steady state trough concentration (day 84) after a fixed, weight (WT) or BSA-based dose for patient populations with $\leqq 10^{th}$ (50.4 kg) (FIG. **14**A) or $\geqq 90^{th}$ (88.5 kg) (FIG. **14**B)WT values.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

A "fixed" or "flat" dose of a therapeutic agent herein refers to a dose that is administered to a human patient without regard for the weight (WT) or body surface area (BSA) of the patient. The fixed or flat dose is therefore not provided as a mg/kg dose or a mg/m^2 dose, but rather as an absolute amount of the therapeutic agent.

A "loading" dose herein generally comprises an initial dose of a therapeutic agent administered to a patient, and is followed by one or more maintenance dose(s) thereof. Generally, a single loading dose is administered, but multiple loading doses are contemplated herein. Usually, the amount of loading dose(s) administered exceeds the amount of the maintenance dose(s) administered and/or the loading dose(s) are administered more frequently than the maintenance dose(s), so as to achieve the desired steady-state concentration of the therapeutic agent earlier than can be achieved with the maintenance dose(s).

A "maintenance" dose herein refers to one or more doses of a therapeutic agent administered to the patient over a treatment period. Usually, the maintenance doses are administered at spaced treatment intervals, such as approximately every week, approximately every 2 weeks, approximately every 3 weeks, or approximately every 4 weeks.

A "HER receptor" is a receptor protein tyrosine kinase which belongs to the HER receptor family and includes EGFR, HER2, HER3 and HER4 receptors. The HER receptor will generally comprise an extracellular domain, which may bind an HER ligand and/or dimerize with another HER receptor molecule; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The HER receptor may be a "native sequence" HER receptor or an "amino acid sequence variant" thereof. Preferably the HER receptor is native sequence human HER receptor.

The terms "ErbB1," "HER1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881-914 (1987), including naturally occurring mutant forms thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS* (*USA*) 87:4207-4211 (1990)). erbBI refers to the gene encoding the EGFR protein product.

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to human HER2 protein described, for example, in Semba et al., *PNAS* (USA) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). The term "erbB2" refers to the gene encoding human ErbB2 and "neu" refers to the gene encoding rat p185^{neu}. Preferred HER2 is native sequence human HER2.

The extracellular domain of HER2 comprises four domains: "Domain I" (amino acid residues from about 1-195; SEQ ID NO:19), "Domain II" (amino acid residues from about 196-319; SEQ ID NO:20), "Domain III" (amino acid residues from about 320-488: SEQ ID NO:21), and "Domain IV" (amino acid residues from about 489-630; SEQ ID NO:22) (residue numbering without signal peptide). See Garrett et al. *Mol. Cell.* 11: 495-505 (2003), Cho et al. *Nature* 421: 756-760 (2003), Franklin et al. *Cancer Cell* 5:317-328 (2004), and Plowman et al. *Proc. Natl. Acad. Sci.* 90:1746-1750 (1993), as well as FIG. **1** herein.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS* (USA) 86:9193-9197 (1989).

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-475 (1993), including isoforms thereof, e.g., as disclosed in WO99/19488, published Apr. 22, 1999.

By "HER ligand" is meant a polypeptide which binds to and/or activates a HER receptor. The HER ligand of particular interest herein is a native sequence human HER ligand such as epidermal growth factor (EGF) (Savage et al., *J. Biol. Chem.* 247:7612-7621 (1972)); transforming growth factor alpha (TGF-α) (Marquardt et al., *Science* 223:1079-1082 (1984)); amphiregulin also known as schwanoma or keratinocyte autocrine growth factor (Shoyab et al. *Science* 243:1074-1076 (1989); Kimura et al. *Nature* 348:257-260 (1990); and Cook et al. *Mol. Cell. Biol.* 11:2547-2557 (1991)); betacellulin (Shing et al., *Science* 259:1604-1607 (1993); and Sasada et al. *Biochem. Biophys. Res. Commun.* 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., *Science* 251:936-939 (1991)); epiregulin (Toyoda et al., *J. Biol. Chem.* 270:7495-7500 (1995); and Komurasaki et al. *Oncogene* 15:2841-2848 (1997)); a heregu-

lin (see below); neuregulin-2 (NRG-2) (Carraway et al., *Nature* 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., *Proc. Natl. Acad. Sci.* 94:9562-9567 (1997)); neuregulin-4 (NRG-4) (Harari et al. *Oncogene* 18:2681-89 (1999)); and cripto (CR-1) (Kannan et al. *J. Biol. Chem.* 272(6):3330-3335 (1997)). HER ligands which bind EGFR include EGF, TGF-α, amphiregulin, betacellulin, HB-EGF and epiregulin. HER ligands which bind HER3 include heregulins. HER ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3, NRG-4, and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide encoded by the heregulin gene product as disclosed in U.S. Pat. No. 5,641,869, or Marchionni et al., *Nature,* 362:312-318 (1993). Examples of heregulins include heregulin-α, heregulin-β1, heregulin-β2 and heregulin-β3 (Holmes et al., *Science,* 256:1205-1210 (1992); and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. *Cell* 69:205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. *Cell* 72:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al., *Nature,* 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho et al. *J. Biol. Chem.* 270:14523-14532 (1995)); γ-heregulin (Schaefer et al. *Oncogene* 15:1385-1394 (1997)).

A "HER dimer" herein is a noncovalently associated dimer comprising at least two HER receptors. Such complexes may form when a cell expressing two or more HER receptors is exposed to an HER ligand and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20):14661-14665 (1994), for example. Examples of such HER dimers include EGFR-HER2, HER2-HER3 and HER3-HER4 heterodimers. Moreover, the HER dimer may comprise two or more HER2 receptors combined with a different HER receptor, such as HER3, HER4 or EGFR. Other proteins, such as a cytokine receptor subunit (e.g. gp130) may be associated with the dimer.

A "HER inhibitor" is an agent which interferes with HER activation or function. Examples of HER inhibitors include HER antibodies (e.g. EGFR, HER2, HER3, or HER4 antibodies); EGFR-targeted drugs; small molecule HER antagonists; HER tyrosine kinase inhibitors; HER2 and EGFR dual tyrosine kinase inhibitors such as lapatinib/GW572016; antisense molecules (see, for example, WO2004/87207); and/or agents that bind to, or interfere with function of, downstream signaling molecules, such as MAPK or Akt (see FIG. **5**). Preferably, the HER inhibitor is an antibody or small molecule which binds to a HER receptor.

A "HER antibody" is an antibody that binds to a HER receptor. Optionally, the HER antibody further interferes with HER activation or function. Preferably, the HER antibody binds to the HER2 receptor. A HER2 antibody of particular interest herein is pertuzumab. Another example of a HER2 antibody is trastuzumab. Examples of EGFR antibodies include cetuximab and ABX0303.

"HER activation" refers to activation, or phosphorylation, of any one or more HER receptors. Generally, HER activation results in signal transduction (e.g. that caused by an intracellular kinase domain of a HER receptor phosphorylating tyrosine residues in the HER receptor or a substrate polypeptide). HER activation may be mediated by HER ligand binding to a HER dimer comprising the HER receptor of interest. HER ligand binding to a HER dimer may activate a kinase domain of one or more of the HER receptors in the dimer and thereby results in phosphorylation of tyrosine residues in one or more of the HER receptors and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s), such as Akt or MAPK intracellular kinases. See, FIG. **5**, for example.

"Phosphorylation" refers to the addition of one or more phosphate group(s) to a protein, such as a HER receptor, or substrate thereof.

An antibody which "inhibits HER dimerization" is an antibody which inhibits, or interferes with, formation of a HER dimer. Preferably, such an antibody binds to HER2 at the heterodimeric binding site thereof. The most preferred dimerization inhibiting antibody herein is pertuzumab or MAb 2C4. Binding of 2C4 to the heterodimeric binding site of HER2 is illustrated in FIG. **4**. Other examples of antibodies which inhibit HER dimerization include antibodies which bind to EGFR and inhibit dimerization thereof with one or more other HER receptors (for example EGFR monoclonal antibody 806, MAb 806, which binds to activated or "untethered" EGFR; see Johns et al., *J. Biol. Chem.* 279(29):30375-30384 (2004)); antibodies which bind to HER3 and inhibit dimerization thereof with one or more other HER receptors; and antibodies which bind to HER4 and inhibit dimerization thereof with one or more other HER receptors.

An antibody which "inhibits HER dimerization more effectively than trastuzumab" is one which reduces or eliminates HER dimers more effectively (for example at least about 2-fold more effectively) than trastuzumab. Preferably, such an antibody inhibits HER2 dimerization at least about as effectively as an antibody selected from the group consisting of murine monoclonal antibody 2C4, a Fab fragment of murine monoclonal antibody 2C4, pertuzumab, and a Fab fragment of pertuzumab. One can evaluate HER dimerization inhibition by studying HER dimers directly, or by evaluating HER activation, or downstream signaling, which results from HER dimerization, and/or by evaluating the antibody-HER2 binding site, etc. Assays for screening for antibodies with the ability to inhibit HER dimerization more effectively than trastuzumab are described in Agus et al. *Cancer Cell* 2: 127-137 (2002) and WO01/00245 (Adams et al.). By way of example only, one may assay for inhibition of HER dimerization by assessing, for example, inhibition of HER dimer formation (see, e.g., FIG. **1**A-B of Agus et al. *Cancer Cell* 2: 127-137 (2002); and WO01/00245); reduction in HER ligand activation of cells which express HER dimers (WO01/00245 and FIG. **2**A-B of Agus et al. *Cancer Cell* 2: 127-137 (2002), for example); blocking of HER ligand binding to cells which express HER dimers (WO01/00245, and FIG. **2**E of Agus et al. *Cancer Cell* 2: 127-137 (2002), for example); cell growth inhibition of cancer cells (e.g. MCF7, MDA-MD-134, ZR-75-1, MD-MB-175, T47D cells) which express HER dimers in the presence (or absence) of HER ligand (WO01/00245 and FIGS. **3**A-D of Agus et al. *Cancer Cell* 2: 127-137 (2002), for instance); inhibition of downstream signaling (for instance, inhibition of HRG-dependent AKT phosphorylation or inhibition of HRG- or TGFα-dependent MAPK phosphorylation) (see, WO01/00245, and FIG. **2**C-D of Agus et al. *Cancer Cell* 2: 127-137 (2002), for example). One may also assess whether the antibody inhibits HER dimerization by studying the antibody-HER2 binding site, for instance, by evaluating a structure or model, such as a crystal structure, of the antibody bound to HER2 (See, for example, Franklin et al. *Cancer Cell* 5:317-328 (2004)).

A "heterodimeric binding site" on HER2, refers to a region in the extracellular domain of HER2 that contacts, or interfaces with, a region in the extracellular domain of EGFR, HER3 or HER4 upon formation of a dimer therewith. The region is found in Domain II of HER2. Franklin et al. *Cancer Cell* 5:317-328 (2004).

The HER2 antibody may "inhibit HRG-dependent AKT phosphorylation" and/or inhibit "HRG- or TGFα-dependent MAPK phosphorylation" more effectively (for instance at least 2-fold more effectively) than trastuzumab (see Agus et al. *Cancer Cell* 2: 127-137 (2002) and WO01/00245, by way of example).

The HER2 antibody may be one which does "not inhibit HER2 ectodomain cleavage" (Molina et al. *Cancer Res.* 61:4744-4749(2001)).

A HER2 antibody that "binds to a heterodimeric binding site" of HER2, binds to residues in domain II (and optionally also binds to residues in other of the domains of the HER2 extracellular domain, such as domains I and III), and can sterically hinder, at least to some extent, formation of a HER2-EGFR, HER2-HER3, or HER2-HER4 heterodimer. Franklin et al. *Cancer Cell* 5:317-328 (2004) characterize the HER2-pertuzumab crystal structure, deposited with the RCSB Protein Data Bank (ID Code IS78), illustrating an exemplary antibody that binds to the heterodimeric binding site of HER2.

An antibody that "binds to domain II" of HER2 binds to residues in domain II and optionally residues in other domain(s) of HER2, such as domains I and III. Preferably the antibody that binds to domain II binds to the junction between domains I, II and III of HER2.

A "native sequence" polypeptide is one which has the same amino acid sequence as a polypeptide (e.g., HER receptor or HER ligand) derived from nature. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments, so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope(s), except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. Such monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones or recombinant DNA clones. It should be understood that the selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, the monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler et al., *Nature,* 256:495 (1975); Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681, (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage display technologies (see, e.g., Clackson et al., *Nature,* 352:624-628 (1991); Marks et al., *J. Mol. Biol.,* 222: 581-597 (1991); Sidhu et al., *J. Mol. Biol.* 338(2):299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5):1073-1093 (2004); Fellouse, *Proc. Nat. Acad. Sci. USA* 101(34):12467-12472 (2004); and Lee et al. *J. Immunol. Methods* 284(1-2):119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggemann et al., *Year in Immuno.,* 7:33 (1993); U.S. Pat. Nos. 5,545,806; 5,569,825; 5,591,669 (all of GenPharm); U.S. Pat. No. 5,545,807; WO 1997/17852; U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks et al., *Bio/Technology,* 10: 779-783 (1992); Lonberg et al., *Nature,* 368: 856-859 (1994); Morrison, *Nature,* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnology,* 14: 845-851 (1996); Neuberger, *Nature Biotechnology,* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.,* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, Ape etc) and human constant region sequences.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragment(s).

An "intact antibody" herein is one which comprises two antigen binding regions, and an Fc region. Preferably, the intact antibody has one or more effector functions.

Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes". There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different

classes of antibodies are called a, δ, ϵ, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

Antibody "effector functions" refer to those biological activities attributable to an Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500, 362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS* (USA) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)), and regulates homeostasis of immunoglobulins.

"Complement dependent cytotoxicity" or "CDC" refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (e.g. an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994). HER2 antibody scFv fragments are described in WO93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a variable heavy domain (V_H) connected to a variable light domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

"Humanized" forms of non-human (e.g., rodent) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992).

Humanized HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 or trastuzumab (HERCEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337 expressly incorporated herein by reference; humanized 520C9 (WO93/21319); and humanized 2C4 antibodies as described herein.

For the purposes herein, "trastuzumab," "HERCEPTIN®," and "huMAb4D5-8" refer to an antibody comprising the light and heavy chain amino acid sequences in SEQ ID NOS. 15 and 16, respectively.

Herein, "pertuzumab" and "OMNITARG™" refer to an antibody comprising the light and heavy chain amino acid sequences in SEQ ID NOS. 13 and 14, respectively.

Differences between trastuzumab and pertuzumab functions are illustrated in FIG. **6**.

A "naked antibody" is an antibody that is not conjugated to a heterologous molecule, such as a cytotoxic moiety or radiolabel.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

An "affinity matured" antibody is one with one or more alterations in one or more hypervariable regions thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. *Bio/Technology* 10:779-783 (1992) describes affinity maturation by V_H and V_L domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. *Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994); Schier et al. *Gene* 169:147-155 (1995); Yelton et al. *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al., *J. Mol. Biol.* 226:889-896 (1992).

The term "main species antibody" herein refers to the antibody structure in a composition which is the quantitatively predominant antibody molecule in the composition. In one embodiment, the main species antibody is a HER2 antibody, such as an antibody that binds to Domain II of HER2, antibody that inhibits HER dimerization more effectively than trastuzumab, and/or an antibody which binds to a heterodimeric binding site of HER2. The preferred embodiment herein of the main species antibody is one comprising the

variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4, and most preferably comprising the light chain and heavy chain amino acid sequences in SEQ ID Nos. 13 and 14 (pertuzumab).

An "amino acid sequence variant" antibody herein is an antibody with an amino acid sequence which differs from a main species antibody. Ordinarily, amino acid sequence variants will possess at least about 70% homology with the main species antibody, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with the main species antibody. The amino acid sequence variants possess substitutions, deletions, and/or additions at certain positions within or adjacent to the amino acid sequence of the main species antibody. Examples of amino acid sequence variants herein include acidic variant (e.g. deamidated antibody variant), basic variant, the antibody with an amino-terminal leader extension (e.g. VHS−) on one or two light chains thereof, antibody with a C-terminal lysine residue on one or two heavy chains thereof, etc, and includes combinations of variations to the amino acid sequences of heavy and/or light chains. The antibody variant of particular interest herein is the antibody comprising an amino-terminal leader extension on one or two light chains thereof, optionally further comprising other amino acid sequence and/or glycosylation differences relative to the main species antibody.

A "glycosylation variant" antibody herein is an antibody with one or more carbohydrate moeities attached thereto which differ from one or more carbohydate moieties attached to a main species antibody. Examples of glycosylation variants herein include antibody with a G1 or G2 oligosaccharide structure, instead a G0 oligosaccharide structure, attached to an Fc region thereof, antibody with one or two carbohydrate moieties attached to one or two light chains thereof, antibody with no carbohydrate attached to one or two heavy chains of the antibody, etc, and combinations of glycosylation alterations.

Where the antibody has an Fc region, an oligosaccharide structure such as that shown in FIG. **9** herein may be attached to one or two heavy chains of the antibody, e.g. at residue 299 (298, Eu numbering of residues). For pertuzumab, G0 was the predominant oligosaccharide structure, with other oligosaccharide structures such as G0-F, G-1, Man5, Man6, G1-1, G1(1-6), G1(1-3) and G2 being found in lesser amounts in the pertuzumab composition.

Unless indicated otherwise, a "G1 oligosaccharide structure" herein includes G-1, G1-1, G1(1-6) and G1(1-3) structures.

An "amino-terminal leader extension" herein refers to one or more amino acid residues of the amino-terminal leader sequence that are present at the amino-terminus of any one or more heavy or light chains of an antibody. An exemplary amino-terminal leader extension comprises or consists of three amino acid residues, VHS, present on one or both light chains of an antibody variant.

A "deamidated" antibody is one in which one or more asparagine residues thereof has been derivitized, e.g. to an aspartic acid, a succinimide, or an iso-aspartic acid.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma (including medulloblastoma and retinoblastoma), sarcoma (including liposarcoma and synovial cell sarcoma), neuroendocrine tumors (including carcinoid tumors, gastrinoma, and islet cell cancer), mesothelioma, schwannoma (including acoustic neuroma), meningioma, adenocarcinoma, melanoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer (including metastatic breast cancer), colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, testicular cancer, esophagael cancer, tumors of the biliary tract, as well as head and neck cancer.

Herein, a "patient" is a human patient. The patient may be a "cancer patient," i.e. one who is suffering or at risk for suffering from one or more symptoms of cancer, or other patient who could benefit from therapy with a HER antibody.

A "biological sample" refers to a sample, generally cells or tissue derived from a biological source.

A "patient sample" refers to a sample obtained from a patient, such as a cancer patient.

A "tumor sample" herein is a sample derived from, or comprising tumor cells from, a patient's tumor. Examples of tumor samples herein include, but are not limited to, tumor biopsies, circulating tumor cells, circulating plasma proteins, ascitic fluid, primary cell cultures or cell lines derived from tumors or exhibiting tumor-like properties, as well as preserved tumor samples, such as formalin-fixed, paraffin-embedded tumor samples or frozen tumor samples.

A "fixed" tumor sample is one which has been histologically preserved using a fixative.

A "formalin-fixed" tumor sample is one which has been preserved using formaldehyde as the fixative.

An "embedded" tumor sample is one surrounded by a firm and generally hard medium such as paraffin, wax, celloidin, or a resin. Embedding makes possible the cutting of thin sections for microscopic examination or for generation of tissue microarrays (TMAs).

A "paraffin-embedded" tumor sample is one surrounded by a purified mixture of solid hydrocarbons derived from petroleum.

Herein, a "frozen" tumor sample refers to a tumor sample which is, or has been, frozen.

A cancer or biological sample which "displays HER expression, amplification, or activation" is one which, in a diagnostic test, expresses (including overexpresses) a HER receptor, has amplified HER gene, and/or otherwise demonstrates activation or phosphorylation of a HER receptor. Such activation can be determined directly (e.g. by measuring HER phosphorylation) or indirectly (e.g. by gene expression profiling or by detecting HER heterodimers, as described herein).

A cancer with "HER receptor overexpression or amplification" is one which has significantly higher levels of a HER receptor protein or gene compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. HER receptor overexpression or amplification may be determined in a diagnostic or prognostic assay by evaluating increased levels of the HER protein present on the surface of a cell (e.g. via an immunohistochemistry assay; IHC). Alternatively, or additionally, one may measure levels of HER-encoding nucleic acid in the cell, e.g. via fluorescent in situ hybridization (FISH; see WO98/45479 published October, 1998), southern blotting, or polymerase chain reaction (PCR)

techniques, such as quantitative real time PCR (qRT-PCR). One may also study HER receptor overexpression or amplification by measuring shed antigen (e.g., HER extracellular domain) in a biological fluid such as serum (see, e.g., U.S. Pat. No. 4,933,294 issued Jun. 12, 1990; WO91/05264 published Apr. 18, 1991; U.S. Pat. No. 5,401,638 issued Mar. 28, 1995; and Sias et al. *J. Immunol. Methods* 132: 73-80 (1990)). Aside from the above assays, various in vivo assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, e.g. a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, e.g. by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

Conversely, a cancer which "does not overexpress or amplify HER receptor" is one which does not have higher than normal levels of HER receptor protein or gene compared to a noncancerous cell of the same tissue type. Antibodies that inhibit HER dimerization, such as pertuzumab, may be used to treat cancer which does not overexpress or amplify HER2 receptor.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially a HER expressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of HER expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13.

Examples of "growth inhibitory" antibodies are those which bind to HER2 and inhibit the growth of cancer cells overexpressing HER2. Preferred growth inhibitory HER2 antibodies inhibit growth of SK-BR-3 breast tumor cells in cell culture by greater than 20%, and preferably greater than 50% (e.g. from about 50% to about 100%) at an antibody concentration of about 0.5 to 30 μg/ml, where the growth inhibition is determined six days after exposure of the SK-BR-3 cells to the antibody (see U.S. Pat. No. 5,677,171 issued Oct. 14, 1997). The SK-BR-3 cell growth inhibition assay is described in more detail in that patent and hereinbelow. The preferred growth inhibitory antibody is a humanized variant of murine monoclonal antibody 4D5, e.g., trastuzumab.

An antibody which "induces apoptosis" is one which induces programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which overexpresses the HER2 receptor. Preferably the cell is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SK-BR-3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an annexin binding assay using BT474 cells (see below). Examples of HER2 antibodies that induce apoptosis are 7C2 and 7F3.

The "epitope 2C4" is the region in the extracellular domain of HER2 to which the antibody 2C4 binds. In order to screen for antibodies which bind to the 2C4 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Preferably the antibody blocks 2C4's binding to HER2 by about 50% or more. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 2C4 epitope of HER2. Epitope 2C4 comprises residues from Domain II in the extracellular domain of HER2. 2C4 and pertuzumab binds to the extracellular domain of HER2 at the junction of domains I, II and III. Franklin et al. *Cancer Cell* 5:317-328 (2004).

The "epitope 4D5" is the region in the extracellular domain of HER2 to which the antibody 4D5 (ATCC CRL 10463) and trastuzumab bind. This epitope is close to the transmembrane domain of HER2, and within Domain IV of HER2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of HER2 (e.g. any one or more residues in the region from about residue 529 to about residue 625, inclusive of the HER2 ECD, residue numbering including signal peptide).

The "epitope 7C2/7F3" is the region at the N terminus, within Domain I, of the extracellular domain of HER2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on HER2 (e.g. any one or more of residues in the region from about residue 22 to about residue 53 of the HER2 ECD, residue numbering including signal peptide).

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disease as well as those in which the disease is to be prevented. Hence, the patient to be treated herein may have been diagnosed as having the disease or may be predisposed or susceptible to the disease.

The term "effective amount" refers to an amount of a drug effective to treat cancer in the patient. The effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. The effective amount may extend progression free survival (e.g. as measured by Response Evaluation Criteria for Solid

Tumors, RECIST, or CA-125 changes), result in an objective response (including a partial response, PR, or complete response, CR), increase overall survival time, and/or improve one or more symptoms of cancer (e.g. as assessed by FOSI).

"Overall survival" refers to the patient remaining alive for a defined period of time, such as 1 year, 5 years, etc, e.g., from the time of diagnosis or treatment.

"Progression free survival" refers to the patient remaining alive, without the cancer getting worse. An "objective response" refers to a measurable response, including complete response (CR) or partial response (PR).

By "complete response" or "complete remission" is intended the disappearance of all signs of cancer in response to treatment. This does not always mean the cancer has been cured.

"Partial response" refers to a decrease in the size of one or more tumors or lesions, or in the extent of cancer in the body, in response to treatment.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; TLK 286 (TELCYTA™); acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; bisphosphonates, such as clodronate; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, e.g., Agnew, *Chem Intl. Ed. Engl.,* 33: 183-186 (1994)) and anthracyclines such as annamycin, AD 32, alcarubicin, daunorubicin, dexrazoxane, DX-52-1, epirubicin, GPX-100, idarubicin, KRN5500, menogaril, dynemicin, including dynemicin A, an esperamicin, neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, liposomal doxorubicin, and deoxydoxorubicin), esorubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, and zorubicin; folic acid analogues such as denopterin, pteropterin, and trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, and floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, and testolactone; anti-adrenals such as aminoglutethimide, mitotane, and trilostane; folic acid replenisher such as folinic acid (leucovorin); aceglatone; anti-folate anti-neoplastic agents such as ALIMTA®, LY231514 pemetrexed, dihydrofolate reductase inhibitors such as methotrexate, anti-metabolites such as 5-fluorouracil (5-FU) and its prodrugs such as UFT, S—I and capecitabine, and thymidylate synthase inhibitors and glycinamide ribonucleotide formyltransferase inhibitors such as raltitrexed (TOMUDEX®, TDX); inhibitors of dihydropyrimidine dehydrogenase such as eniluracil; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids and taxanes, e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Ill.), and TAXOTERE® docetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; platinum; platinum analogs or platinum-based analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine (VELBAN®); etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); vinca alkaloid; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids such as retinoic acid; pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovorin.

Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON® toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-

imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

An "antimetabolite chemotherapeutic agent" is an agent which is structurally similar to a metabolite, but can not be used by the body in a productive manner. Many antimetabolite chemotherapeutic agents interfere with the production of the nucleic acids, RNA and DNA. Examples of antimetabolite chemotherapeutic agents include gemcitabine (GEMZAR®), 5-fluorouracil (5-FU), capecitabine (XELODA™), 6-mercaptopurine, methotrexate, 6-thioguanine, pemetrexed, raltitrexed, arabinosylcytosine ARA-C cytarabine (CYTOSAR-U®), dacarbazine (DTIC-DOME®), azocytosine, deoxycytosine, pyridmidene, fludarabine (FLUDARA®), cladrabine, 2-deoxy-D-glucose etc. The preferred antimetabolite chemotherapeutic agent is gemcitabine.

"Gemcitabine" or "2'-deoxy-2',2'-difluorocytidine monohydrochloride (b-isomer)" is a nucleoside analogue that exhibits antitumor activity. The empirical formula for gemcitabine HCl is C9H11F2N3O4.HCl. Gemcitabine HCl is sold by Eli Lilly under the trademark GEMZAR®.

A "platinum-based chemotherapeutic agent" comprises an organic compound which contains platinum as an integral part of the molecule. Examples of platinum-based chemotherapeutic agents include carboplatin, cisplatin, and oxaliplatinum.

By "platinum-based chemotherapy" is intended therapy with one or more platinum-based chemotherapeutic agents, optionally in combination with one or more other chemotherapeutic agents.

By "platinum resistant" cancer is meant that the cancer patient has progressed while receiving platinum-based chemotherapy (i.e. the patient is "platinum refractory"), or the patient has progressed within 12 months (for instance, within 6 months) after completing a platinum-based chemotherapy regimen;

An "anti-angiogenic agent" refers to a compound which blocks, or interferes with to some degree, the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or antibody that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. The preferred anti-angiogenic factor herein is an antibody that binds to vascular endothelial growth factor (VEGF), such as bevacizumab (AVASTIN®).

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-αand -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-αand TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

As used herein, the term "EGFR-targeted drug" refers to a therapeutic agent that binds to EGFR and, optionally, inhibits EGFR activation. Examples of such agents include antibodies and small molecules that bind to EGFR. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225 or Cetuximab; ERBUTIX®) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.); antibodies that bind type II mutant EGFR (U.S. Pat. No. 5,212,290); humanized and chimeric antibodies that bind EGFR as described in U.S. Pat. No. 5,891,996; and human antibodies that bind EGFR, such as ABX-EGF (see WO98/50433, Abgenix); EMD 55900 (Stragliotto et al. *Eur. J. Cancer* 32A:636-640 (1996)); and mAb 806 or humanized mAb 806 (Johns et al., *J. Biol. Chem.* 279(29):30375-30384 (2004)). The anti-EGFR antibody may be conjugated with a cytotoxic agent, thus generating an immunoconjugate (see, e.g., EP659,439A2, Merck Patent GmbH). Examples of small molecules that bind to EGFR include ZD1839 or Gefitinib (IRESSA™; Astra Zeneca); CP-358774 or Erlotinib HCL (TARCEVA™; Genentech/OSI); and AG 1478, AG1571 (SU 5271; Sugen).

A "tyrosine kinase inhibitor" is a molecule which inhibits tyrosine kinase activity of a tyrosine kinase such as a HER receptor. Examples of such inhibitors include the EGFR-targeted drugs noted in the preceding paragraph; small molecule HER2 tyrosine kinase inhibitor such as TAK165 available from Takeda; dual-HER inhibitors such as EKB-569 (available from Wyeth) which preferentially binds EGFR but inhibits both HER2 and EGFR-overexpressing cells; GW572016 (available from Glaxo) an oral HER2 and EGFR tyrosine kinase inhibitor; PKI-166 (available from Novartis); pan-HER inhibitors such as canertinib (CI-1033; Pharmacia); Raf-1 inhibitors such as antisense agent ISIS-5132 available from ISIS Pharmaceuticals which inhibits Raf-1 signaling; non-HER targeted TK inhibitors such as Imatinib mesylate (Gleevac™) available from Glaxo; MAPK extracellular regulated kinase 1 inhibitor CI-1040 (available from Pharmacia); quinazolines, such as PD 153035,4-(3-chloroanilino) quinazoline; pyridopyrimidines; pyrimidopyrimidines; pyrrolopyrimidines, such as CGP 59326, CGP 60261 and CGP 62706; pyrazolopyrimidines, 4-(phenylamino)-7H-pyrrolo [2,3-d]pyrimidines; curcumin (diferuloyl methane, 4,5-bis (4-fluoroanilino)phthalimide); tyrphostines containing nitrothiophene moieties; PD-0183805 (Warner-Lamber); antisense molecules (e.g. those that bind to HER-encoding nucleic acid); quinoxalines (U.S. Pat. No. 5,804,396); try-

phostins (U.S. Pat. No. 5,804,396); ZD6474 (Astra Zeneca); PTK-787 (Novartis/Schering AG); pan-HER inhibitors such as CI-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); Imatinib mesylate (Gleevac; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); CI-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Sugen); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-1C11 (Imclone); or as described in any of the following patent publications: U.S. Pat. No. 5,804,396; WO99/09016 (American Cyanimid); WO98/43960 (American Cyanamid); WO97/38983 (Warner Lambert); WO99/06378 (Warner Lambert); WO99/06396 (Warner Lambert); WO96/30347 (Pfizer, Inc); WO96/33978 (Zeneca); WO96/3397 (Zeneca); and WO96/33980 (Zeneca).

An "autoimmune disease" herein is a disease or disorder arising from and directed against an individual's own tissues or a co-segregate or manifestation thereof or resulting condition therefrom. Examples of autoimmune diseases or disorders include, but are not limited to arthritis (rheumatoid arthritis such as acute arthritis, chronic rheumatoid arthritis, gouty arthritis, acute gouty arthritis, chronic inflammatory arthritis, degenerative arthritis, infectious arthritis, Lyme arthritis, proliferative arthritis, psoriatic arthritis, vertebral arthritis, and juvenile-onset rheumatoid arthritis, osteoarthritis, arthritis chronica progrediente, arthritis deformans, polyarthritis chronica primaria, reactive arthritis, and ankylosing spondylitis), inflammatory hyperproliferative skin diseases, psoriasis such as plaque psoriasis, gutatte psoriasis, pustular psoriasis, and psoriasis of the nails, atopy including atopic diseases such as hay fever and Job's syndrome, dermatitis including contact dermatitis, chronic contact dermatitis, allergic dermatitis, allergic contact dermatitis, dermatitis herpetiformis, and atopic dermatitis, x-linked hyper IgM syndrome, urticaria such as chronic allergic urticaria and chronic idiopathic urticaria, including chronic autoimmune urticaria, polymyositis/dermatomyositis, juvenile dermatomyositis, toxic epidermal necrolysis, scleroderma (including systemic scleroderma), sclerosis such as systemic sclerosis, multiple sclerosis (MS) such as spino-optical MS, primary progressive MS (PPMS), and relapsing remitting MS (RRMS), progressive systemic sclerosis, atherosclerosis, arteriosclerosis, sclerosis disseminata, and ataxic sclerosis, inflammatory bowel disease (IBD) (for example, Crohn's disease, autoimmune-mediated gastrointestinal diseases, colitis such as ulcerative colitis, colitis ulcerosa, microscopic colitis, collagenous colitis, colitis polyposa, necrotizing enterocolitis, and transmural colitis, and autoimmune inflammatory bowel disease), pyoderma gangrenosum, erythema nodosum, primary sclerosing cholangitis, episcleritis), respiratory distress syndrome, including adult or acute respiratory distress syndrome (ARDS), meningitis, inflammation of all or part of the uvea, iritis, choroiditis, an autoimmune hematological disorder, rheumatoid spondylitis, sudden hearing loss, IgE-mediated diseases such as anaphylaxis and allergic and atopic rhinitis, encephalitis such as Rasmussen's encephalitis and limbic and/or brainstem encephalitis, uveitis, such as anterior uveitis, acute anterior uveitis, granulomatous uveitis, nongranulomatous uveitis, phacoantigenic uveitis, posterior uveitis, or autoimmune uveitis, glomerulonephritis (GN) with and without nephrotic syndrome such as chronic or acute glomerulonephritis such as primary GN, immune-mediated GN, membranous GN (membranous nephropathy), idiopathic membranous GN or idiopathic membranous nephropathy, membrano- or membranous proliferative GN (MPGN), including Type I and Type II, and rapidly progressive GN, allergic conditions and responses, allergic reaction, eczema including allergic or atopic eczema, asthma such as asthma bronchiale, bronchial asthma, and auto-immune asthma, conditions involving infiltration of T cells and chronic inflammatory responses, immune reactions against foreign antigens such as fetal A-B-O blood groups during pregnancy, chronic pulmonary inflammatory disease, autoimmune myocarditis, leukocyte adhesion deficiency, systemic lupus erythematosus (SLE) or systemic lupus erythematodes such as cutaneous SLE, subacute cutaneous lupus erythematosus, neonatal lupus syndrome (NLE), lupus erythematosus disseminatus, lupus (including nephritis, cerebritis, pediatric, non-renal, extra-renal, discoid, alopecia), juvenile onset (Type I) diabetes mellitus, including pediatric insulin-dependent diabetes mellitus (IDDM), adult onset diabetes mellitus (Type II diabetes), autoimmune diabetes, idiopathic diabetes insipidus, immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes, tuberculosis, sarcoidosis, granulomatosis including lymphomatoid granulomatosis, Wegener's granulomatosis, agranulocytosis, vasculitides, including vasculitis (including large vessel vasculitis (including polymyalgia rheumatica and giant cell (Takayasu's) arteritis), medium vessel vasculitis (including Kawasaki's disease and polyarteritis nodosa/periarteritis nodosa), microscopic polyarteritis, CNS vasculitis, necrotizing, cutaneous, or hypersensitivity vasculitis, systemic necrotizing vasculitis, and ANCA-associated vasculitis, such as Churg-Strauss vasculitis or syndrome (CSS)), temporal arteritis, aplastic anemia, autoimmune aplastic anemia, Coombs positive anemia, Diamond Blackfan anemia, hemolytic anemia or immune hemolytic anemia including autoimmune hemolytic anemia (AIHA), pernicious anemia (anemia perniciosa), Addison's disease, pure red cell anemia or aplasia (PRCA), Factor VIII deficiency, hemophilia A, autoimmune neutropenia, pancytopenia, leukopenia, diseases involving leukocyte diapedesis, CNS inflammatory disorders, multiple organ injury syndrome such as those secondary to septicemia, trauma or hemorrhage, antigen-antibody complex -mediated diseases, anti-glomerular basement membrane disease, anti-phospholipid antibody syndrome, allergic neuritis, Bechet's or Behcet's disease, Castleman's syndrome, Goodpasture's syndrome, Reynaud's syndrome, Sjogren's syndrome, Stevens-Johnson syndrome, pemphigoid such as pemphigoid bullous and skin pemphigoid, pemphigus (including pemphigus vulgaris, pemphigus foliaceus, pemphigus mucus-membrane pemphigoid, and pemphigus erythematosus), autoimmune polyendocrinopathies, Reiter's disease or syndrome, immune complex nephritis, antibody-mediated nephritis, neuromyelitis optica, polyneuropathies, chronic neuropathy such as IgM polyneuropathies or IgM-mediated neuropathy, thrombocytopenia (as developed by myocardial infarction patients, for example), including thrombotic thrombocytopenic purpura (TTP), post-transfusion purpura (PTP), heparin-induced thrombocytopenia, and autoimmune or immune-mediated thrombocytopenia such as idiopathic thrombocytopenic purpura (ITP) including chronic or acute ITP, autoimmune disease of the testis and ovary including autoimune orchitis and oophoritis, primary hypothyroidism, hypoparathyroidism, autoimmune endocrine diseases including thyroiditis such as autoimmune thyroiditis, Hashimoto's disease, chronic thyroiditis (Hashimoto's thyroiditis), or subacute thyroiditis, autoimmune thyroid disease, idiopathic hypothyroidism, Grave's disease, polyglandular syndromes such as autoimmune polyglandular syndromes (or polyglandular endocrinopathy syndromes), paraneoplastic syndromes, including neurologic paraneoplastic syndromes such as Lambert-Eaton myasthenic syndrome or Eaton-Lambert syndrome, stiff-man or stiff-person syndrome, encephalomyelitis such as allergic encephalomyelitis or encephalomyelitis allergica and experimental aller-

gic encephalomyelitis (EAE), myasthenia gravis such as thymoma-associated myasthenia gravis, cerebellar degeneration, neuromyotonia, opsoclonus or opsoclonus myoclonus syndrome (OMS), and sensory neuropathy, multifocal motor neuropathy, Sheehan's syndrome, autoimmune hepatitis, chronic hepatitis, lupoid hepatitis, giant cell hepatitis, chronic active hepatitis or autoimmune chronic active hepatitis, lymphoid interstitial pneumonitis (LIP), bronchiolitis obliterans (non-transplant) vs NSIP, Guillain-Barré syndrome, Berger's disease (IgA nephropathy), idiopathic IgA nephropathy, linear IgA dermatosis, primary biliary cirrhosis, pneumonocirrhosis, autoimmune enteropathy syndrome, Celiac disease, Coeliac disease, celiac sprue (gluten enteropathy), refractory sprue, idiopathic sprue, cryoglobulinemia, amylotrophic lateral sclerosis (ALS; Lou Gehrig's disease), coronary artery disease, autoimmune ear disease such as autoimmune inner ear disease (AIED), autoimmune hearing loss, opsoclonus myoclonus syndrome (OMS), polychondritis such as refractory or relapsed polychondritis, pulmonary alveolar proteinosis, amyloidosis, scleritis, a non-cancerous lymphocytosis, a primary lymphocytosis, which includes monoclonal B cell lymphocytosis (e.g., benign monoclonal gammopathy and monoclonal garnmopathy of undetermined significance, MGUS), peripheral neuropathy, paraneoplastic syndrome, channelopathies such as epilepsy, migraine, arrhythmia, muscular disorders, deafness, blindness, periodic paralysis, and channelopathies of the CNS, autism, inflammatory myopathy, focal segmental glomerulosclerosis (FSGS), endocrine ophthalmopathy, uveoretinitis, chorioretinitis, autoimmune hepatological disorder, fibromyalgia, multiple endocrine failure, Schmidt's syndrome, adrenalitis, gastric atrophy, presenile dementia, demyelinating diseases such as autoimmune demyelinating diseases and chronic inflammatory demyelinating polyneuropathy, diabetic nephropathy, Dressier's syndrome, alopecia greata, CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia), male and female autoimmune infertility, mixed connective tissue disease, Chagas' disease, rheumatic fever, recurrent abortion, farmer's lung, erythema multiforme, post-cardiotomy syndrome, Cushing's syndrome, bird -fancier's lung, allergic granulomatous angiitis, benign lymphocytic angiitis, Alport's syndrome, alveolitis such as allergic alveolitis and fibrosing alveolitis, interstitial lung disease, transfusion reaction, leprosy, malaria, leishmaniasis, kypanosomiasis, schistosomiasis, ascariasis, aspergillosis, Sampter's syndrome, Caplan's syndrome, dengue, endocarditis, endomyocardial fibrosis, diffuse interstitial pulmonary fibrosis, interstitial lung fibrosis, pulmonary fibrosis, idiopathic pulmonary fibrosis, cystic fibrosis, endophthalniitis, erythema elevatum et diutinum, erythroblastosis fetalis, eosinophilic faciitis, Shulman's syndrome, Felty's syndrome, flariasis, cyclitis such as chronic cyclitis, heterochronic cyclitis, iridocyclitis (acute or chronic), or Fuch's cyclitis, Henoch-Schonlein purpura, human immunodeficiency virus (HIV) infection, echovirus infection, cardiomyopathy, Alzheimer's disease, parvovirus infection, rubella virus infection, post-vaccination syndromes, congenital rubella infection, Epstein-Barr virus infection, mumps, Evan's syndrome, autoimmune gonadal failure, Sydenham's chorea, post-streptococcal nephritis, thromboangitis ubiterans, thyrotoxicosis, tabes dorsalis, chorioiditis, giant cell polymyalgia, endocrine ophthamopathy, chronic hypersensitivity pneumonitis, keratoconjunctivitis sicca, epidemic keratoconjunctivitis, idiopathic nephritic syndrome, minimal change nephropathy, benign familial and ischemia-reperfusion injury, retinal autoimmunity, joint inflammation, bronchitis, chronic obstructive airway disease, silicosis, aphthae, aphthous stomatitis, arteriosclerotic disorders, aspermiogenese, autoimmune hemolysis, Boeck's disease, cryoglobulinemia, Dupuytren's contracture, endophthalmia phacoanaphylactica, enteritis allergica, erythema nodosum leprosum, idiopathic facial paralysis, chronic fatigue syndrome, febris rheumatica, Hamman-Rich's disease, sensoneural hearing loss, haemoglobinuria paroxysmatica, hypogonadism, ileitis regionalis, leucopenia, mononucleosis infectiosa, traverse myelitis, primary idiopathic myxedema, nephrosis, ophthalmia symphatica, orchitis granulomatosa, pancreatitis, polyradiculitis acuta, pyoderma gangrenosum, Quervain's thyreoiditis, acquired spenic atrophy, infertility due to antispermatozoan antobodies, non-malignant thymoma, vitiligo, SCID and Epstein-Barr virus-associated diseases, acquired immune deficiency syndrome (AIDS), parasitic diseases such as Lesihmania, toxic-shock syndrome, food poisoning, conditions involving infiltration of T cells, leukocyte-adhesion deficiency, immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes, diseases involving leukocyte diapedesis, multiple organ injury syndrome, antigen-antibody complex-mediated diseases, antiglomerular basement membrane disease, allergic neuritis, autoimmune polyendocrinopathies, oophoritis, primary myxedema, autoimmune atrophic gastritis, sympathetic ophthalmia, rheumatic diseases, mixed connective tissue disease, nephrotic syndrome, insulitis, polyendocrine failure, peripheral neuropathy, autoimmune polyglandular syndrome type I, adult-onset idiopathic hypoparathyroidism (AOIH), alopecia totalis, dilated cardiomyopathy, epidermolisis bullosa acquisita (EBA), hemochromatosis, myocarditis, nephrotic syndrome, primary sclerosing cholangitis, purulent or nonpurulent sinusitis, acute or chronic sinusitis, ethmoid, frontal, maxillary, or sphenoid sinusitis, an eosinophil-related disorder such as eosinophilia, pulmonary infiltration eosinophilia, eosinophilia-myalgia syndrome, Loffler's syndrome, chronic eosinophilic pneumonia, tropical pulmonary eosinophilia, bronchopneumonic aspergillosis, aspergilloma, or granulomas containing eosinophils, anaphylaxis, seronegative spondyloarthritides, polyendocrine autoimmune disease, sclerosing cholangitis, sclera, episclera, chronic mucocutaneous candidiasis, Bruton's syndrome, transient hypogammaglobulinemia of infancy, Wiskott-Aldrich syndrome, ataxia telangiectasia, autoimmune disorders associated with collagen disease, rheumatism, neurological disease, lyniphadenitis, ischemic re-perfusion disorder, reduction in blood pressure response, vascular dysfunction, antgiectasis, tissue injury, cardiovascular ischemia, hyperalgesia, cerebral ischemia, and disease accompanying vascularization, allergic hypersensitivity disorders, glomerulonephritides, reperfusion injury, reperfusion injury of myocardial or other tissues, dermatoses with acute inflammatory components, acute purulent meningitis or other central nervous system inflammatory disorders, ocular and orbital inflammatory disorders, granulocyte transfusion-associated syndromes, cytokine-induced toxicity, narcolepsy, acute serious inflammation, chronic intractable inflammation, pyelitis, pneumonocirrhosis, diabetic retinopathy, diabetic large-artery disorder, endarterial hyperplasia, peptic ulcer, valvulitis, and endometriosis.

A "benign hyperproliferative disorder" is meant a state in a patient that relates to cell proliferation and which is recognized as abnormal by members of the medical community. An abnormal state is characterized by a level of a property that is statistically different from the level observed in organisms not suffering from the disorder. Cell proliferation refers to growth or extension by multiplication of cells and includes cell divi-

sion. The rate of cell proliferation may be measured by counting the number of cells produced in a given unit of time. Examples of benign hyperproliferative disorders include psoriasis and polyps.

A "respiratory disease" involves the respiratory system and includes chronic bronchitis, asthma including acute asthma and allergic asthma, cystic fibrosis, bronchiectasis, allergic or other rhinitis or sinusitis, α1-antitrypsin deficiency, coughs, pulmonary emphysema, pulmonary fibrosis or hyper -reactive airways, chronic obstructive pulmonary disease, and chronic obstructive lung disorder.

"Psoriasis" is a condition characterized by the eruption of circumscribed, discrete and confluent, reddish, silvery-scaled maculopapules. Psoriatic lesions generally occur predominantly on the elbows, knees, scalp, and trunk, and microscopically show characteristic parakerotosis and elongation of rete ridges. The term includes the various forms of psoriasis, including erythrodermic, pustular, moderate-severe and recalcitrant forms of the disease.

"Endometriosis" refers to the ectopic occurrence of endometrial tissue, frequently forming cysts containing altered blood.

The term "vascular disease or disorder" herein refers to the various diseases or disorders which impact the vascular system, including the cardiovascular system. Examples of such diseases include arteriosclerosis, vascular reobstruction, atherosclerosis, postsurgical vascular stenosis, restenosis, vascular occlusion or carotid obstructive disease, coronary artery disease, angina, small vessel disease, hypercholesterolemia, hypertension, and conditions involving abnormal proliferation or function of vascular epithelial cells.

The term "stenosis" refers to narrowing or stricture of a hollow passage (e,g, a duct or canal) in the body.

The term "vascular stenosis" refers to occlusion or narrowing of blood vessels. Vascular stenosis often results from fatty deposit (as in the case of atherosclerosis) or excessive migration and proliferation of vascular smooth muscle cells and endothelial cells. Arteries are particularly susceptible to stenosis. The term "stenosis" as used herein specifically includes initial stenosis and restenosis.

The term "restenosis" refers to recurrence of stenosis after treatment of initial stenosis with apparent success. For example, "restenosis" in the context of vascular stenosis, refers to the reoccurrence of vascular stenosis after it has been treated with apparent success, e.g. by removal of fatty deposit by angioplasty (e.g. percutaneous transluminal coronary angioplasty), direction coronary atherectomy or stent etc. One of the contributing factors in restenosis is intimal hyperplasia. The term "intimal hyperplasia", used interchangeably with "neointimal hyperplasia" and "neointima formation", refers to thickening of the inner most layer of blood vessels, intima, as a consequence of excessive proliferation and migration of vascular smooth muscle cells and endothelial cells. The various changes taking place during restenosis are often collectively referred to as "vascular wall remodeling."

The terms "balloon angioplasty" and "percutaneous transluminal coronary angioplasty" (PTCA) are often used interchangeably, and refer to a non-surgical catheter-based treatment for removal of plaque from the coronary artery. Stenosis or restenosis often lead to hypertension as a result of increased resistance to blood flow.

The term "hypertension" refers to abnormally high blood pressure, i.e. beyond the upper value of the normal range.

"Polyps" refers to a mass of tissue that bulges or projects outward or upward from the normal surface level, thereby being macroscopically visible as a hemispheroidal, speroidal, or irregular moundlike structure growing from a relatively broad base or a slender stalk. Examples include colon, rectal and nasal polyps.

"Fibroadenoma" references a benign neoplasm derived from glandular epithelium, in which there is a conspicuous stroma of proliferating fibroblasts and connective tissue elements. This commonly occurs in breast tissue.

"Asthma" is a condition which results in difficulty in breathing. Bronchial asthma refers to a condition of the lungs in which there is widespread narrowing of airways, which may be due to contraction (spasm) of smooth muscle, edema of the mucosa, or mucus in the lumen of the bronchi and bronchioles.

"Bronchitis" refers to inflammation of the mucous membrane of the bronchial tubes.

For the purposes herein, a "vial" refers to a container which holds a therapeutic agent. The vial may be sealed by a stopper pierceable by a syringe. Generally, the vial is formed from glass material. The therapeutic agent in the vial can be in various states including liquid, lyophilized, frozen etc.

A "package insert" refers to instructions customarily included in commercial packages of therapeutic agents, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

II. Production of Antibodies

A description follows as to exemplary techniques for the production of HER antibodies used in accordance with the present invention. The HER antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of HER or a portion thereof, containing the desired epitope. Alternatively, cells expressing HER at their cell surface (e.g. NIH-3T3 cells transformed to overexpress HER2; or a carcinoma cell line such as SK-BR-3 cells, see Stancovski et al. *PNAS* (USA) 88:8691-8695 (1991)) can be used to generate antibodies. Other forms of HER receptor useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N -hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross -linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Various methods for making monoclonal antibodies herein are available in the art. For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); and Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130:151-188 (1992).

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy chain and light chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; and Morrison, et al., *Proc. Natl. Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized Antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of

known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three -dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

WO01/00245 describes production of exemplary humanized HER2 antibodies which bind HER2 and block ligand activation of a HER receptor. The humanized antibody of particular interest herein blocks EGF, TGF-α and/or HRG mediated activation of MAPK essentially as effectively as murine monoclonal antibody 2C4 (or a Fab fragment thereof) and/or binds HER2 essentially as effectively as murine monoclonal antibody 2C4 (or a Fab fragment thereof). The humanized antibody herein may, for example, comprise nonhuman hypervariable region residues incorporated into a human variable heavy domain and may further comprise a framework region (FR) substitution at a position selected from the group consisting of 69H, 71H and 73H utilizing the variable domain numbering system set forth in Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). In one embodiment, the humanized antibody comprises FR substitutions at two or all of positions 69H, 71H and 73H.

An exemplary humanized antibody of interest herein comprises variable heavy domain complementarity determining residues GFTFTDYTMX, where X is preferrably D or S (SEQ ID NO:7); DVNPNSGGSIYNQRFKG (SEQ ID NO:8); and/or NLGPSFYFDY (SEQ ID NO:9), optionally comprising amino acid modifications of those CDR residues, e.g. where the modifications essentially maintain or improve affinity of the antibody. For example, the antibody variant of interest may have from about one to about seven or about five amino acid substitutions in the above variable heavy CDR sequences. Such antibody variants may be prepared by affinity maturation, e.g., as described below. The most preferred humanized antibody comprises the variable heavy domain amino acid sequence in SEQ ID NO:4.

The humanized antibody may comprise variable light domain complementarity determining residues KASQDVSIGVA (SEQ ID NO:10); SASYX^{1}X^{2}X^3, where X^1 is preferably R or L, X^2 is preferably Y or E, and X^3 is preferably T or S (SEQ ID NO:11); and/or QQYYIYPYT (SEQ ID NO:12), e.g. in addition to those variable heavy domain CDR residues in the preceding paragraph. Such humanized antibodies optionally comprise amino acid modifications of the above CDR residues, e.g. where the modifications essentially maintain or improve affinity of the antibody. For example, the antibody variant of interest may have from about one to about seven or about five amino acid substitutions in the above variable light CDR sequences. Such antibody variants may be prepared by affinity maturation, e.g., as described below. The most preferred humanized antibody comprises the variable light domain amino acid sequence in SEQ ID NO:3.

The present application also contemplates affinity matured antibodies which bind HER2 and block ligand activation of a HER receptor. The parent antibody may be a human antibody or a humanized antibody, e.g., one comprising the variable light and/or heavy sequences of SEQ ID Nos. 3 and 4, respectively (i.e. variant 574). The affinity matured antibody preferably binds to HER2 receptor with an affinity superior to that of murine 2C4 or variant 574 (e.g. from about two or about four fold, to about 100 fold or about 1000 fold improved affinity, e.g. as assessed using a HER2-extracellular domain (ECD) ELISA). Exemplary variable heavy CDR residues for substitution include H28, H30, H34, H35, H64, H96, H99, or combinations of two or more (e.g. two, three, four, five, six, or seven of these residues). Examples of variable light CDR residues for alteration include L28, L50, L53, L56, L91, L92, L93, L94, L96, L97 or combinations of two or more (e.g. two to three, four, five or up to about ten of these residues).

Various forms of the humanized antibody or affinity matured antibody are contemplated. For example, the humanized antibody or affinity matured antibody may be an antibody fragment, such as a Fab, which is optionally conjugated with one or more cytotoxic agent(s) in order to generate an immunoconjugate. Alternatively, the humanized antibody or affinity matured antibody may be an intact antibody, such as an intact IgG1 antibody. The preferred intact IgG1 antibody comprises the light chain sequence in SEQ ID NO:13 and the heavy chain sequence in SEQ ID NO:14.

(iv) Human Antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germline mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and U.S. Pat. Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty et al., *Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M113 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage

genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., *Nature,* 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., *J. Mol. Biol.* 222:581-597-(1991), or Griffith et al., *EMBO J.* 12:725-734 (1993). See, also, U.S. Pat. Nos. 5,565,332 and 5,573,905.

As discussed above, human antibodies may also be generated by in vitro activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

Human HER2 antibodies are described in U.S. Pat. No. 5,772,997 issued Jun. 30, 1998 and WO 97/00271 published Jan. 3, 1997.

(v) Antibody Fragments

Various techniques have been developed for the production of antibody fragments comprising one or more antigen binding regions. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the HER2 protein. Other such antibodies may combine a HER2 binding site with binding site(s) for EGFR, HER3 and/or HER4. Alternatively, a HER2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the HER2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express HER2. These antibodies possess a HER2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

WO 96/16673 describes a bispecific HER2/FcγRIII antibody and U.S. Pat. No. 5,837,234 discloses a bispecific HER2/FcγRI antibody IDM1 (Osidem). A bispecific HER2/Fcα antibody is shown in WO98/02463. U.S. Pat. No. 5,821,337 teaches a bispecific HER2/CD3 antibody. MDX-210 is a bispecific HER2-FcγRIII Ab.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in U.S. Pat. No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the CH3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increas-

ing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5): 1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

(vii) Other Amino Acid Sequence Modifications

Amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells *Science,* 244:1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed antibody variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn

TABLE 1-continued

Original Residue	Exemplary Substitutions	Preferred Substitutions
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in *Biochemistry*, second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gin (O)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human HER2. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that lacks fucose attached to an Fc region of the antibody are described in US Pat Appl No US 2003/0157108 A1, Presta, L. See also US 2004/0093621 A1 (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetylglucosamine (GlcNAc) in the carbohydrate attached to an Fc region of the antibody are referenced in WO03/011878, Jean-Mairet et al. and U.S. Pat. No. 6,602,684, Umana et al. Antibodies with at least one galactose residue in the oligosaccharide attached to an Fc region of the antibody are reported in WO97/30087, Patel et al. See, also, WO98/58964 (Raju, S.) and WO99/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof.

It may be desirable to modify the antibody of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-

2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

WO00/42072 (Presta, L.) describes antibodies with improved ADCC function in the presence of human effector cells, where the antibodies comprise amino acid substitutions in the Fc region thereof. Preferably, the antibody with improved ADCC comprises substitutions at positions 298, 333, and/or 334 of the Fc region. Preferably the altered Fc region is a human IgG1 Fc region comprising or consisting of substitutions at one, two or three of these positions.

Antibodies with altered C1q binding and/or complement dependent cytotoxicity (CDC) are described in WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.). The antibodies comprise an amino acid substitution at one or more of amino acid positions 270, 322, 326, 327, 329, 313, 333 and/or 334 of the Fc region thereof.

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Pat. No. 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

Antibodies with improved binding to the neonatal Fc receptor (FcRn), and increased half-lives, are described in WO00/42072 (Presta, L.). These antibodies comprise a Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. For example, the Fc region may have substitutions at one or more of positions 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434. The preferred Fc region-comprising antibody variant with improved FcRn binding comprises amino acid substitutions at one, two or three of positions 307, 380 and 434 of the Fc region thereof.

Engineered antibodies with three or more (preferably four) functional antigen binding sites are also contemplated (US Appln No. US2002/0004587 A1, Miller et al.).

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

(viii) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. One may further select antibodies with certain biological characteristics, as desired.

To identify an antibody which blocks ligand activation of a HER receptor, the ability of the antibody to block HER ligand binding to cells expressing the HER receptor (e.g. in conjugation with another HER receptor with which the HER receptor of interest forms a HER hetero-oligomer) may be determined. For example, cells naturally expressing, or transfected to express, HER receptors of the HER hetero-oligomer may be incubated with the antibody and then exposed to labeled HER ligand. The ability of the antibody to block ligand binding to the HER receptor in the HER hetero-oligomer may then be evaluated.

For example, inhibition of HRG binding to MCF7 breast tumor cell lines by HER2 antibodies may be performed using monolayer MCF7 cultures on ice in a 24-well-plate format essentially as described in WO01/00245. HER2 monoclonal antibodies may be added to each well and incubated for 30 minutes. ^{125}I-labeled $rHRG\beta1_{177-224}$ (25 pm) may then be added, and the incubation may be continued for 4 to 16 hours. Dose response curves may be prepared and an IC_{50} value may be calculated for the antibody of interest. In one embodiment, the antibody which blocks ligand activation of a HER receptor will have an IC_{50} for inhibiting HRG binding to MCF7 cells in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG binding to MCF7 cells in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

Alternatively, or additionally, the ability of an antibody to block HER ligand-stimulated tyrosine phosphorylation of a HER receptor present in a HER hetero-oligomer may be assessed. For example, cells endogenously expressing the HER receptors or transfected to expressed them may be incubated with the antibody and then assayed for HER ligand-dependent tyrosine phosphorylation activity using an anti-phosphotyrosine monoclonal (which is optionally conjugated with a detectable label). The kinase receptor activation assay described in U.S. Pat. No. 5,766,863 is also available for determining HER receptor activation and blocking of that activity by an antibody.

In one embodiment, one may screen for an antibody which inhibits HRG stimulation of p180 tyrosine phosphorylation in MCF7 cells essentially as described in WO01/00245. For example, the MCF7 cells may be plated in 24-well plates and monoclonal antibodies to HER2 may be added to each well and incubated for 30 minutes at room temperature; then $rHRG\beta1_{177-244}$ may be added to each well to a final concentration of 0.2 nM, and the incubation may be continued for 8 minutes. Media may be aspirated from each well, and reactions may be stopped by the addition of 100 μl of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 μl) may be electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (at 1 μg/ml) immunoblots may be developed, and the intensity of the predominant reactive band at M_r~180,000 may be quantified by reflectance densitometry. The antibody selected will preferably significantly inhibit HRG stimulation of p180 tyrosine phosphorylation to about 0-35% of control in this assay. A dose-response curve for inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry may be prepared and an IC_{50} for the antibody of interest may be calculated. In one embodiment, the antibody which blocks ligand activation of a HER receptor will have an IC_{50} for inhibiting HRG stimulation of p180 tyrosine phosphorylation in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG stimulation of p180 tyrosine phosphorylation in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

One may also assess the growth inhibitory effects of the antibody on MDA-MB-175 cells, e.g, essentially as described in Schaefer et al. *Oncogene* 15:1385-1394 (1997). According to this assay, MDA-MB-175 cells may treated with a HER2 monoclonal antibody (10 μg/mL) for 4 days and stained with crystal violet. Incubation with a HER2 antibody may show a growth inhibitory effect on this cell line similar to that displayed by monoclonal antibody 2C4. In a further embodi-

ment, exogenous HRG will not significantly reverse this inhibition. Preferably, the antibody will be able to inhibit cell proliferation of MDA-MB-175 cells to a greater extent than monoclonal antibody 4D5 (and optionally to a greater extent than monoclonal antibody 7F3), both in the presence and absence of exogenous HRG.

In one embodiment, the HER2 antibody of interest may block heregulin dependent association of HER2 with HER3 in both MCF7 and SK-BR-3 cells as determined in a co-immunoprecipitation experiment such as that described in WO01/00245 substantially more effectively than monoclonal antibody 4D5, and preferably substantially more effectively than monoclonal antibody 7F3.

To identify growth inhibitory HER2 antibodies, one may screen for antibodies which inhibit the growth of cancer cells which overexpress HER2. In one embodiment, the growth inhibitory antibody of choice is able to inhibit growth of SK-BR-3 cells in cell culture by about 20-100% and preferably by about 50-100% at an antibody concentration of about 0.5 to 30 μg/ml. To identify such antibodies, the SK-BR-3 assay described in U.S. Pat. No. 5,677,171 can be performed. According to this assay, SK-BR-3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin streptomycin. The SK-BR-3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 0.5 to 30 μg/ml of the HER2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SK-BR-3 cells by about 20-100% or about 50-100% may be selected as growth inhibitory antibodies. See U.S. Pat. No. 5,677,171 for assays for screening for growth inhibitory antibodies, such as 4D5 and 3E8.

In order to select for antibodies which induce apoptosis, an annexin binding assay using BT474 cells is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the monoclonal antibody. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^+ binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies. In addition to the annexin binding assay, a DNA staining assay using BT474 cells is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay. See WO98/17797 for assays for screening for antibodies which induce apoptosis, such as 7C2 and 7F3.

To screen for antibodies which bind to an epitope on HER2 bound by an antibody of interest, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed to assess whether the antibody cross-blocks binding of an antibody, such as 2C4 or pertuzumab, to HER2. Alternatively, or additionally, epitope mapping can be performed by methods known in the art and/or one can study the antibody-HER2 structure (Franklin et al. *Cancer Cell* 5:317-328 (2004)) to see what domain(s) of HER2 is/are bound by the antibody.

(ix) Pertuzumab Compositions

In one embodiment of a HER2 antibody composition, the composition comprises a mixture of a main species pertuzumab antibody and one or more variants thereof. The preferred embodiment herein of a pertuzumab main species antibody is one comprising the variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4, and most preferably comprising a light chain amino acid sequence selected from SEQ ID No. 13 and 17, and a heavy chain amino acid sequence selected from SEQ ID No. 14 and 18 (including deamidated and/or oxidized variants of those sequences). In one embodiment, the composition comprises a mixture of the main species pertuzumab antibody and an amino acid sequence variant thereof comprising an amino-terminal leader extension. Preferably, the amino-terminal leader extension is on a light chain of the antibody variant (e.g. on one or two light chains of the antibody variant). The main species HER2 antibody or the antibody variant may be an full length antibody or antibody fragment (e.g. Fab of F(ab')2 fragments), but preferably both are full length antibodies. The antibody variant herein may comprise an amino-terminal leader extension on any one or more of the heavy or light chains thereof. Preferably, the amino-terminal leader extension is on one or two light chains of the antibody. The amino-terminal leader extension preferably comprises or consists of VHS−. Presence of the amino-terminal leader extension in the composition can be detected by various analytical techniques including, but not limited to, N-terminal sequence analysis, assay for charge heterogeneity (for instance, cation exchange chromatography or capillary zone electrophoresis), mass spectrometry, etc. The amount of the antibody variant in the composition generally ranges from an amount that constitutes the detection limit of any assay (preferably N-terminal sequence analysis) used to detect the variant to an amount less than the amount of the main species antibody. Generally, about 20% or less (e.g. from about 1% to about 15%, for instance from 5% to about 15%) of the antibody molecules in the composition comprise an amino-terminal leader extension. Such percentage amounts are preferably determined using quantitative N-terminal sequence analysis or cation exchange analysis (preferably using a high-resolution, weak cation -exchange column, such as a PROPAC WCX-10™ cation exchange column). Aside from the amino -terminal leader extension variant, further amino acid sequence alterations of the main species antibody and/or variant are contemplated, including but not limited to an antibody comprising a C-terminal lysine residue on one or both heavy chains thereof, a deamidated antibody variant, etc.

Moreover, the main species antibody or variant may further comprise glycosylation variations, non-limiting examples of which include antibody comprising a G1 or G2 oligosaccharide structure attached to the Fc region thereof, antibody comprising a carbohydrate moiety attached to a light chain thereof (e.g. one or two carbohydrate moieties, such as glucose or galactose, attached to one or two light chains of the antibody, for instance attached to one or more lysine residues), antibody comprising one or two non -glycosylated heavy chains, or antibody comprising a sialidated oligosaccharide attached to one or two heavy chains thereof etc.

The composition may be recovered from a genetically engineered cell line, e.g. a Chinese Hamster Ovary (CHO) cell line expressing the HER2 antibody, or may be prepared by peptide synthesis.

(x) Immunoconjugates

The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. a small molecule toxin or an enzymatically active toxin of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, a maytansine (U.S. Pat. No. 5,208,020), a trichothene, and CC 1065 are also contemplated herein.

In one preferred embodiment of the invention, the antibody is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody (Chari et al. *Cancer Research* 52: 127-131 (1992)) to generate a maytansinoid-antibody immunoconjugate.

Another immunoconjugate of interest comprises an antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^1, α_2^1, α_3^1, N-acetyl-γ_1^1, PSAG and θ^1_1 (Hinman et al. *Cancer Research* 53: 3336-3342 (1993) and Lode et al. *Cancer Research* 58: 2925-2928 (1998)). See, also, U.S. Pat. Nos. 5,714,586; 5,712,374; 5,264,586; and 5,773,001 expressly incorporated herein by reference.

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), *momordica charantia* inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published Oct. 28, 1993.

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

A variety of radioactive isotopes are available for the production of radioconjugated HER2 antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol)propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al. *Cancer Research* 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis.

Other immunoconjugates are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences,* 16th edition, Oslo, A., Ed., (1980).

The antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA,* 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published Oct. 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19)1484 (1989).

III. Selecting Patients for Therapy

The patient herein is optionally subjected to a diagnostic test prior to therapy. For example, the diagnostic test may evaluate HER (e.g. HER2 or EGFR) expression (including overexpression), amplification, and/or activation (including phosphorylation or dimerization).

Generally, if a diagnostic test is performed, a sample may be obtained from a patient in need of therapy. Where the subject has cancer, the sample is generally a tumor sample. In the preferred embodiment, the tumor sample is from an ovarian cancer, peritoneal cancer, fallopian tube cancer, metastatic breast cancer (MBC), non-small cell lung cancer (NSCLC), prostate cancer, or colorectal cancer tumor sample.

It is noted however, that various other non-malignant therapeutic indications for HER antibodies are described herein. Where the patient is to be treated for those non-malignant indications, a suitable sample can be obtained from the patient and subjected to a diagnostic assay as described herein.

The biological sample herein may be a fixed sample, e.g. a formalin fixed, paraffin-embedded (FFPE) sample, or a frozen sample.

According to one embodiment of the invention herein, the patient selected for therapy has a tumor displaying HER (and preferably HER2) activation. In one embodiment, the extent of HER (or HER2) activation in cancer cells significantly exceeds the level of activation of that receptor in non-cancerous cells of the same tissue type. Such excessive activation may result from overexpression of the HER receptor and/or greater than normal levels of a HER ligand available for activating the HER receptor in the cancer cells. Such excessive activation may cause and/or be caused by the malignant state of a cancer cell. In some embodiments, the cancer will be subjected to a diagnostic or prognostic assay to determine whether amplification and/or overexpression of a HER receptor is occurring which results in such excessive activation of the HER receptor. Alternatively, or additionally, the cancer may be subjected to a diagnostic or prognostic assay to determine whether amplification and/or overexpression a HER ligand is occurring in the cancer which attributes to excessive activation of the receptor. In a subset of such cancers, excessive activation of the receptor may result from an autocrine stimulatory pathway. Various assays for determining HER activation will be described in more detail below.

(i) HER Dimers

Tumors samples can be assessed for the presence of HER dimers, as indicating HER or HER2 activation. Any method known in the art may be used to detect HER2 dimers, such as EGFR-HER2, HER2-HER3, in tumors. Several preferred methods are described below. These methods detect noncovalent protein-protein interactions or otherwise indicate proximity between proteins of interest.

Immunoaffinity-based methods, such as immunoprecipitation or ELISA, may be used to detect HER dimers. In one embodiment, HER2 antibodies are used to immunoprecipitate complexes comprising HER2 from tumor cells, and the resulting immunoprecipitant is then probed for the presence of EGFR or HER3 by immunoblotting. In another embodiment, EGFR or HER3 antibodies may be used for the immunoprecipitation step and the immunoprecipitant then probed with HER2 antibodies. In a further embodiment, HER ligands specific to EGFR, HER3, EGFR/HER2 complexes or HER2/HER3 complexes may be used to precipitate complexes, which are then probed for the presence of HER2. For example, ligands may be conjugated to avidin and complexes purified on a biotin column.

In other embodiments, such as ELISA or antibody "sandwich"-type assays, antibodies to HER2 are immobilized on a solid support, contacted with tumor cells or tumor cell lysate, washed, and then exposed to antibody against EGFR or HER3. Binding of the latter antibody, which may be detected directly or by a secondary antibody conjugated to a detectable label, indicates the presence of heterodimers. In certain embodiments, EGFR or HER3 antibody is immobilized, and HER2 antibody is used for the detection step. In other embodiments HER ligands may be used in place of, or in combination with HER antibodies.

Chemical or UV cross-linking may also be used to covalently join dimers on the surface of living cells. Hunter et al., *Biochem. J.,* 320:847-53. Examples of chemical cross-linkers include dithiobis(succinimidyl) propionate (DSP) and 3,3dithiobis(sulphosuccinimidyl)propionate (DTSSP). In one embodiment, cell extracts from chemically cross-linked tumor cells are analyzed by SDS-PAGE and immunoblotted with antibodies to EGFR and/or HER3. A supershifted band of the appropriate molecular weight most likely represents EGFR-HER2 or HER2-HER3 dimers, as HER2 is the preferred dimerization partner for EGFR and HER3. This result may be confirmed by subsequent immunoblotting with HER2 antibodies.

Fluorescence resonance energy transfer (FRET) may also be used to detect EGFR-HER2 or HER2-HER3 dimers. FRET detects protein conformational changes and protein-protein interactions in vivo and in vitro based on the transfer of energy from a donor fluorophore to an acceptor fluorophore. Selvin, *Nat. Struct. Biol.* 7:730-34 (2000). Energy transfer takes place only if the donor fluorophore is in sufficient proximity to the acceptor fluorophore. In a typical FRET experiment, two proteins or two sites on a single protein are labeled with different fluorescent probes. One of the probes, the donor probe, is excited to a higher energy state by incident light of a specified wavelength. The donor probe then transmits its energy to the second probe, the acceptor probe, resulting in a reduction in the donor's fluorescence intensity and an increase in the acceptor's fluorescence emission. To measure the extent of energy transfer, the donor's intensity in a sample labeled with donor and acceptor probes is compared with its intensity in a sample labeled with donor probe only. Optionally, acceptor intensity is compared in donor/acceptor and acceptor only samples. Suitable probes are known in the art and include, for example, membrane permeant dyes, such as fluorescein and rhodamine, organic dyes, such as the cyanine dyes, and lanthanide atoms. Selvin, supra. Methods and instrumentation for detecting and measuring energy transfer are also known in the art. Selvin, supra.

FRET-based techniques suitable for detecting and measuring protein-protein interactions in individual cells are also known in the art. For example, donor photobleaching fluorescence resonance energy transfer (pbFRET) microscopy and fluorescence lifetime imaging microscopy (FLIM) may be used to detect the dimerization of cell surface receptors. Selvin, supra; Gadella & Jovin, *J. Cell Biol.,* 129:1543-58 (1995). In one embodiment, pbFRET is used on cells either "in suspension" or "in situ" to detect and measure the formation of EGFR-HER2 or HER2-HER3 dimers, as described in Nagy et al., *Cytometry,* 32:120-131 (1998). These techniques measure the reduction in a donor's fluorescence lifetime due to energy transfer. In a particular embodiment, a flow cytometric Foerster-type FRET technique (FCET) may be used to investigate EGFR-HER2 and HER2-HER3 dimerization, as described in Nagy et al., supra, and Brockhoff et al., *Cytometry,* 44:338-48 (2001).

FRET is preferably used in conjunction with standard immunohistochemical labeling techniques. Kenworthy, *Methods,* 24:289-96 (2001). For example, antibodies conjugated to suitable fluorescent dyes can be used as probes for labeling two different proteins. If the proteins are within proximity of one another, the fluorescent dyes act as donors and acceptors for FRET. Energy transfer is detected by standard means. Energy transfer may be detected by flow cytometric means or by digital microscopy systems, such as confocal microscopy or wide-field fluorescence microscopy coupled to a charge-coupled device (CCD) camera.

In one embodiment of the present invention, HER2 antibodies and either EGFR or HER3 antibodies are directly labeled with two different fluorophores, for example as described in Nagy et al, supra. Tumor cells or tumor cell lysates are contacted with the differentially labeled antibodies, which act as donors and acceptors for FRET in the presence of EGFR-HER2 or HER2-HER3 dimers. Alternatively, unlabeled antibodies against HER2 and either EGFR or HER3 are used along with differentially labeled secondary antibodies that serve as donors and acceptors. See, for

example, Brockhoff et al., supra. Energy transfer is detected and the presence of dimers is determined if the labels are found to be in close proximity.

In other embodiments HER receptor ligands that are specific for HER2 and either HER1 or HER3 are fluorescently labeled and used for FRET studies.

In still other embodiments of the present invention, the presence of dimers on the surface of tumor cells is demonstrated by co-localization of HER2 with either EGFR or HER3 using standard direct or indirect immunofluorescence techniques and confocal laser scanning microscopy. Alternatively, laser scanning imaging (LSI) is used to detect antibody binding and co-localization of HER2 with either EGFR or HER3 in a high-throughput format, such as a microwell plate, as described in Zuck et al, *Proc. Natl. Acad. Sci. USA,* 96:11122-27 (1999).

In further embodiments, the presence of EGFR-HER2 and/or HER2-HER3 dimers is determined by identifying enzymatic activity that is dependent upon the proximity of the dimer components. A HER2 antibody is conjugated with one enzyme and an EGFR or HER3 antibody is conjugated with a second enzyme. A first substrate for the first enzyme is added and the reaction produces a second substrate for the second enzyme. This leads to a reaction with another molecule to produce a detectable compound, such as a dye. The presence of another chemical breaks down the second substrate, so that reaction with the second enzyme is prevented unless the first and second enzymes, and thus the two antibodies, are in close proximity. In a particular embodiment tumor cells or cell lysates are contacted with a HER2 antibody that is conjugated with glucose oxidase and a HER3 or HER1 antibody that is conjugated with horse radish peroxidase. Glucose is added to the reaction, along with a dye precursor, such as DAB, and catalase. The presence of dimers is determined by the development of color upon staining for DAB.

Dimers may also be detected using methods based on the eTag™ assay system (Aclara Bio Sciences, Mountain View, Calif.), as described, for example, in U.S. Patent Application 2001/0049105, published Dec. 6, 2001, both of which are expressly incorporated by reference in their entirety. An eTag™, or "electrophoretic tag," comprises a detectable reporter moiety, such as a fluorescent group. It may also comprise a "mobility modifier," which consists essentially of a moiety having a unique electrophoretic mobility. These moieties allow for separation and detection of the eTag™ from a complex mixture under defined electrophoretic conditions, such as capillary electrophoresis (CE). The portion of the eTag™ containing the reporter moiety and, optionally, the mobility modifier is linked to a first target binding moiety by a cleavable linking group to produce a first binding compound. The first target binding moiety specifically recognizes a particular first target, such as a nucleic acid or protein. The first target binding moiety is not limited in any way, and may be for example, a polynucleotide or a polypeptide. Preferably, the first target binding moiety is an antibody or antibody fragment. Alternatively, the first target binding moiety may be a HER receptor ligand or binding-competent fragment thereof.

The linking group preferably comprises a cleavable moiety, such as an enzyme substrate, or any chemical bond that may be cleaved under defined conditions. When the first target binding moiety binds to its target, the cleaving agent is introduced and/or activated, and the linking group is cleaved, thus releasing the portion of the eTag™ containing the reporter moiety and mobility modifier. Thus, the presence of a "free" eTag™ indicates the binding of the target binding moiety to its target.

Preferably, a second binding compound comprises the cleaving agent and a second target binding moiety that specifically recognizes a second target. The second target binding moiety is also not limited in any way and may be, for example, an antibody or antibody fragment or a HER receptor ligand or binding competent ligand fragment. The cleaving agent is such that it will only cleave the linking group in the first binding compound if the first binding compound and the second binding compound are in close proximity.

In an embodiment of the present invention, a first binding compound comprises an eTag™ in which an antibody to HER2 serves as the first target binding moiety. A second binding compound comprises an antibody to EGFR or HER3 joined to a cleaving agent capable of cleaving the linking group of the eTag™. Preferably the cleaving agent must be activated in order to be able to cleave the linking group. Tumor cells or tumor cell lysates are contacted with the eTag™, which binds to HER2, and with the modified EGFR or HER3 antibody, which binds to EGFR or HER3 on the cell surface. Unbound binding compound is preferable removed, and the cleaving agent is activated, if necessary. If EGFR-HER2 or HER2-HER3 dimers are present, the cleaving agent will cleave the linking group and release the eTag™ due to the proximity of the cleaving agent to the linking group. Free eTag™ may then be detected by any method known in the art, such as capillary electrophoresis.

In one embodiment, the cleaving agent is an activatable chemical species that acts on the linking group. For example, the cleaving agent may be activated by exposing the sample to light.

In another embodiment, the eTag™ is constructed using an antibody to EGFR or HER3 as the first target binding moiety, and the second binding compound is constructed from an antibody to HER2.

In yet another embodiment, the HER dimer is detected using an antibody or other reagent which specifically or preferentially binds to the dimer as compared to binding thereof to either HER receptor in the dimer.

(ii) HER2 Phosphorylation

Immunoprecipitation with EGFR, HER2, or HER3 antibody as discussed in the previous section may optionally be followed by a functional assay for dimers, as an alternative or supplement to immunoblotting. In one embodiment, immunoprecipitation with HER3 antibody is followed by an assay for receptor tyrosine kinase activity in the immunoprecipitant. Because HER3 does not have intrinsic tyrosine kinase activity, the presence of tyrosine kinase activity in the immunoprecipitant indicates that HER3 is most likely associated with HER2. Graus-Porta et al., *EMBO J.,* 16:1647-55 (1997); Klapper et al., *Proc. Natl. Acad. Sci. USA,* 96:4995-5000 (1999). This result may be confirmed by immunoblotting with HER2 antibodies. In another embodiment, immunoprecipitation with HER2 antibody is followed by an assay for EGFR receptor tyrosine kinase activity. In this assay, the immunoprecipitant is contacted with radioactive ATP and a peptide substrate that mimics the in vivo site of transphosphorylation of HER2 by EGFR. Phosphorylation of the peptide indicates co-immunoprecipitation and thus dimerization of EGFR with HER2. Receptor tyrosine kinase activity assays are well known in the art and include assays that detect phosphorylation of target substrates, for example, by phosphotyrosine antibody, and activation of cognate signal transduction pathways, such as the MAPK pathway.

Phosphorylation of HER receptor may be assessed by immunoprecipitation of one or more HER receptors, such as HER2 receptor, and Western blot analysis. For example, positivity is determined by the presence of a phospho-HER2 band

on the gel, using an anti-phosphotyrosine antibody to detect phosphorylated tyrosine residue(s) in the immunoprecipitated HER receptor(s). Anti-phosphotyrosine antibodies are commercially available from PanVera (Madison, Wis.), a subsidiary of Invitrogen, Chemicon International Inc. (Temecula, Calif.), or Upstate Biotechnology (Lake Placid, N.Y.). Negativity is determined by the absence of the band.

In another embodiment, phosphorylation of HER2 (HER2) receptor is assessed by immunohistochemistry using a phospho-specific HER2 antibody (clone PN2A; Thor et al., *J. Clin. Oncol,* 18(18):3230-3239 (2000)).

Other methods for detecting phosphorylation of HER receptor(s) include, but are not limited to, KIRA ELISA (U.S. Pat. Nos. 5,766,863; 5,891,650; 5,914,237; 6,025,145; and 6,287,784), mass spectrometry (comparing size of phosphorylated and non-phosphorylated HER2), and e-tag proximity assay with both a HER (e.g. HER2) antibody and phospho-specific or phospho-tyrosine specific antibody (e.g., using the eTag™ assay kit available from Aclara BioSciences (Mountain View, Calif.). Details of the eTag assay are described hereinabove.

One may also use phospho-specific antibodies in cellular array to detect phosphorylation status in a cellular sample of signal transduction protein (US2003/0190689).

(iii) Gene Expression Profile

In one embodiment, a gene expression analyses can serve as a surrogate for measuring HER phosphorylation or activation directly. This is particularly useful where the sample is a fixed sample (e.g. parrafin-embedded, formalin fixed tumor sample) where HER phosphorylation may be difficult to reliably quantify. According to this method, expression of two or more HER receptors and one or more HER ligand in a sample is evaluated, wherein expression of the two or more HER receptors and one or more HER ligand indicates positive HER phosphorylation or activation in the sample. In one embodiment of this method, expression of betacellulin and/or amphiregulin in the sample can be measured, wherein betacellulin and/or amphiregulin expression indicates positive HER phosphorylation or activation in the sample.

According to this method, a sample from the patient is tested for expression of two or more HER receptors (preferably selected from EGFR, HER2, and HER3) and one or more HER ligands (preferably selected from betacellulin, amphiregulin, epiregulin, and TGF-α, most preferably betacellulin or amphiregulin). For example, the two or more HER receptors may be EGFR and HER2, or HER2 and HER3. Preferably, expression of HER2 and EGFR or HER3, as well as betacellulin or amphiregulin is determined. The sample may be tested for expression of betacellulin or amphiregulin alone, or in combination with testing for expression of two or more HER receptors. Positive expression of the identified gene(s) indicates the patient is a candidate for therapy with a HER antibody, such as pertuzumab. Moreover, positive expression of the gene(s) indicates the patient is more likely to respond favorably to therapy with the HER antibody than a patient who does not have such positive expression.

Various methods for determining expression of mRNA or protein include, but are not limited to, gene expression profiling, polymerase chain reaction (PCR) including quantitative real time PCR (qRT-PCR), microarray analysis, serial analysis of gene expression (SAGE), MassARRAY, Gene Expression Analysis by Massively Parallel Signature Sequencing (MPSS), proteomics, immunohistochemistry (IHC), etc. Preferably mRNA is quantified. Such mRNA analysis is preferably performed using the technique of polymerase chain reaction (PCR), or by microarray analysis. Where PCR is employed, a preferred form of PCR is quantitative real time PCR (qRT-PCR). In one embodiment, expression of one or more of the above noted genes is deemed positive expression if it is at the median or above, e.g. compared to other samples of the same tumor-type. The median expression level can be determined essentially contemporaneously with measuring gene expression, or may have been determined previously.

Various exemplary methods for determining gene expression The steps of a representative protocol for profiling gene expression using fixed, paraffin-embedded tissues as the RNA source, including mRNA isolation, purification, primer extension and amplification are given in various published journal articles (for example: Godfrey et al. *J. Molec. Diagnostics* 2: 84-91 (2000); Specht et al., *Am. J. Pathol.* 158: 419-29 (2001)). Briefly, a representative process starts with cutting about 10 microgram thick sections of paraffin-embedded tumor tissue samples. The RNA is then extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be included, if necessary, and RNA is reverse transcribed using gene specific promoters followed by PCR. Finally, the data are analyzed to identify the best treatment option(s) available to the patient on the basis of the characteristic gene expression pattern identified in the tumor sample examined.

(iv) HER Expression and Amplification

To determine HER expression or amplification in the cancer, various diagnostic/prognostic assays are available. In one embodiment, HER overexpression may be analyzed by IHC, e.g. using the HERCEPTEST® (Dako). Parrafin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a HER2 protein staining intensity criteria as follows:

Score 0 no staining is observed or membrane staining is observed in less than 10% of tumor cells.

Score 1+ a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.

Score 2+ a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score 3+ a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

Those tumors with 0 or 1+ scores for HER2 overexpression assessment may be characterized as not overexpressing HER2, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing HER2.

Tumors overexpressing HER2 may be rated by immunohistochemical scores corresponding to the number of copies of HER2 molecules expressed per cell, and can been determined biochemically:

0=0-10,000 copies/cell,

1+=at least about 200,000 copies/cell,

2+=at least about 500,000 copies/cell,

3+=at least about 2,000,000 copies/cell.

Overexpression of HER2 at the 3+level, which leads to ligand-independent activation of the tyrosine kinase (Hudziak et al., *Proc. Natl. Acad. Sci. USA,* 84:7159-7163 (1987)), occurs in approximately 30% of breast cancers, and in these patients, relapse-free survival and overall survival are diminished (Slamon et al., *Science,* 244:707-712 (1989); Slamon et al., *Science,* 235:177-182 (1987)).

Alternatively, or additionally, FISH assays such as the INFORM™ (sold by Ventana, Ariz.) or PATHVISION™ (Vysis, Ill.) may be carried out on formalin-fixed, paraffin-

embedded tumor tissue to determine the extent (if any) of HER2 amplification in the tumor.

In one embodiment, the cancer will be one which expresses (and may overexpress) EGFR, such expression may be evaluated as for the methods for evaluating HER2 expression as noted above.

HER receptor or HER ligand overexpression or amplification may also be evaluated using an in vivo diagnostic assay, e.g. by administering a molecule (such as an antibody) which binds the molecule to be detected and is tagged with a detectable label (e.g. a radioactive isotope) and externally scanning the patient for localization of the label.

IV. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Lyophilized antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The preferred pertuzumab formulation for therapeutic use comprises 30 mg/mL pertuzumab in 20 mM histidine acetate, 120 mM sucrose, 0.02% polysorbate 20, at pH 6.0. An alternate pertuzumab formulation comprises 25 mg/mL pertuzumab, 10 mM histidine-HCl buffer, 240 mM sucrose, 0.02% polysorbate 20, pH 6.0.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Various drugs which can be combined with the HER antibody are described in the method of treatment section below. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and y ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

V. Treatment and Dosing of HER Antibodies

Examples of various cancers that can be treated with a fixed dose of a HER antibody are listed in the definition section above. Preferred cancer indications include ovarian cancer; peritoneal cancer; fallopian tube cancer; breast cancer, including metastatic breast cancer (MBC); lung cancer, including non-small cell lung cancer (NSCLC); prostate cancer; colorectal cancer; and/or cancer which displays HER expression, amplification and/or activation. In one embodiment, the cancer which is treated is chemotherapy-resistant cancer or platinum-resistant cancer. Administration of fixed dose(s) of the antibody will result in an improvement in the signs or symptoms of cancer.

Aside from cancer, fixed dose(s) of the HER antibodies as disclosed herein may be used to treat various non-malignant diseases or disorders. Such non-malignant diseases or disorders include autoimmune disease (e.g. psoriasis; see definition above); endometriosis; scleroderma; restenosis; polyps such as colon polyps, nasal polyps or gastrointestinal polyps; fibroadenoma; respiratory disease (see definition above); cholecystitis; neurofibromatosis; polycystic kidney disease; inflammatory diseases; skin disorders including psoriasis and dermatitis; vascular disease (see definition above); conditions involving abnormal proliferation of vascular epithelial cells; gastrointestinal ulcers; Menetrier's disease, secreting adenomas or protein loss syndrome; renal disorders; angiogenic disorders; ocular disease such as age related macular degeneration, presumed ocular histoplasmosis syndrome, retinal neovascularization from proliferative diabetic retinopathy, retinal vascularization, diabetic retinopathy, or age related macular degeneration; bone associated pathologies such as osteoarthritis, rickets and osteoporosis; damage following a cerebral ischemic event; fibrotic or edemia diseases such as hepatic cirrhosis, lung fibrosis, carcoidosis, throiditis, hyperviscosity syndrome systemic, Osler Weber-Rendu disease, chronic occlusive pulmonary disease, or edema following burns, trauma, radiation, stroke, hypoxia or ischemia; hypersensitivity reaction of the skin; diabetic retinopathy and diabetic nephropathy; Guillain-Barre syndrome; graft versus host disease or transplant rejection; Paget's disease; bone or joint inflammation; photoaging (e.g. caused by UV radiation of human skin); benign prostatic hypertrophy; certain microbial infections including microbial pathogens selected from adenovirus, hantaviruses, *Borrelia burgdorferi, Yersinia* spp. and *Bordetella pertussis*; thrombus caused by platelet aggregation; reproductive conditions such as endometriosis, ovarian hyperstimulation syndrome, preeclampsia, dysfunctional uterine bleeding, or menometrorrhagia; synovitis; atheroma; acute and chronic nephropathies (including proliferative glomerulonephritis and diabetes-induced renal disease); eczema; hypertrophic scar formation; endotoxic shock and fungal infection; familial adenomatosis polyposis; neu-

rodedenerative diseases (e.g. Alzheimer's disease, AIDS-related dementia, Parkinson's disease, amyotrophic lateral sclerosis, retinitis pigmentosa, spinal muscular atrophy and cerebellar degeneration); myelodysplastic syndromes; aplastic anemia; ischemic injury; fibrosis of the lung, kidney or liver; T-cell mediated hypersensitivity disease; infantile hypertrophic pyloric stenosis; urinary obstructive syndrome; psoriatic arthritis; and Hasimoto's thyroiditis. Preferred non-malignant indications for therapy herein include psoriasis, endometriosis, scleroderma, vascular disease (e.g. restenosis, artherosclerosis, coronary artery disease, or hypertension), colon polyps, fibroadenoma or respiratory disease (e.g. asthma, chronic bronchitis, bronchieactasis or cystic fibrosis).

The HER antibody is administered to a human patient in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

For the prevention or treatment of disease, the fixed dose of HER antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The fixed dose is suitably administered to the patient at one time or over a series of treatments. Preferably, the fixed dose is in the range from about 20 mg to about 2000 mg of the HER antibody. For example, the fixed dose may be approximately 420 mg, approximately 525 mg, approximately 840 mg, or approximately 1050 mg of the HER antibody.

Where a series of fixed doses are administered, these may, for example, be administered approximately every week, approximately every 2 weeks, approximately every 3 weeks, or approximately every 4 weeks, but preferably approximately every 3 weeks. The fixed doses may, for example, continue to be administered until disease progression, adverse event, or other time as determined by the physician. For example, from about two, three, or four, up to about 17 or more fixed doses may be administered.

In one embodiment, one or more loading dose(s) of the antibody are administered, followed by one or more maintenance dose(s) of the antibody. In another embodiment, a plurality of the same fixed dose are administered to the patient.

According to one preferred embodiment of the invention, a fixed dose of HER antibody (e.g. pertuzumab) of approximately 840 mg (loading dose) is administered, followed by one or more doses of approximately 420 mg (maintenance dose(s)) of the antibody. The maintenance doses are preferably administered about every 3 weeks, for a total of at least two doses, up to 17 or more doses.

According to another preferred embodiment of the invention, one or more fixed dose(s) of approximately 1050 mg of the HER antibody (e.g. pertzumab) are administered, for example every 3 weeks. According to this embodiment, one, two or more of the fixed doses are administered, e.g. for up to one year (17 cycles), and longer as desired.

In another embodiment, a fixed dose of approximately 1050 mg of HER2 antibody (e.g. pertuzumab) is administered as a loading dose, followed by one or more maintenance dose(s) of approximately 525 mg of the antibody. About one, two or more maintenance doses may be administered to the patient every 3 weeks according to this embodiment.

Thus, the invention provides a method of treating cancer in a human patient comprising administering at least one fixed dose of pertuzumab to the patient, wherein the fixed dose is approximately 420 mg, approximately 525 mg, approximately 840 mg, or approximately 1050 mg of pertuzumab.

Where the disease is cancer, the patient is preferably treated with a combination of the HER antibody, and one or more chemotherapeutic agent(s). Preferably at least one of the chemotherapeutic agents is an antimetabolite chemotherapeutic agent such as gemcitabine. The combined administration includes coadministration or concurrent administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Thus, the antimetabolite chemotherapeutic agent may be administered prior to, or following, administration of the HER antibody. In this embodiment, the timing between at least one administration of the antimetabolite chemotherapeutic agent and at least one administration of the HER antibody is preferably approximately 1 month or less, and most preferably approximately 2 weeks or less. Alternatively, the antimetabolite chemotherapeutic agent and the HER antibody are administered concurrently to the patient, in a single formulation or separate formulations. Treatment with the combination of the chemotherapeutic agent (e.g. antimetabolite chemotherapeutic agent such as gemcitabine) and the HER antibody (e.g. pertuzumab) may result in a synergistic, or greater than additive, therapeutic benefit to the patient.

An antimetabolite chemotherapeutic agent, if administered, is usually administered at dosages known therefor, or optionally lowered due to combined action of the drugs or negative side effects attributable to administration of the antimetabolite chemotherapeutic agent. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Where the antimetabolite chemotherapeutic agent is gemcitabine, preferably, it is administered at a dose between about 600 mg/m to 1250 mg/m2 (for example approximately 1000 mg/m 2), for instance, on days 1 and 8 of a 3-week cycle.

Aside from the HER antibody and antimetabolite chemotherapeutic agent, other therapeutic regimens may be combined therewith. For example, a second (third, fourth, etc) chemotherapeutic agent(s) may be administered, wherein the second chemotherapeutic agent is either another, different antimetabolite chemotherapeutic agent, or a chemotherapeutic agent that is not an antimetabolite. For example, the second chemotherapeutic agent may be a taxane (such as paclitaxel or docetaxel), capecitabine, or platinum-based chemotherapeutic agent (such as carboplatin, cisplatin, or oxaliplatin), anthracycline (such as doxorubicin, including, liposomal doxorubicin), topotecan, pemetrexed, vinca alkaloid (such as vinorelbine), and TLK 286. "Cocktails" of different chemotherapeutic agents may be administered.

Other therapeutic agents that may be combined with the HER antibody include any one or more of: a second, different HER antibody (for example, a growth inhibitory HER2 antibody such as trastuzumab, or a HER2 antibody which induces apoptosis of a HER2-overexpressing cell, such as 7C2, 7F3 or humanized variants thereof); an antibody directed against a different tumor associated antigen, such as EGFR, HER3, HER4; anti-hormonal compound, e.g., an anti-estrogen compound such as tamoxifen, or an aromatase inhibitor; a cardioprotectant (to prevent or reduce any myocardial dysfunction associated with the therapy); a cytokine; an EGFR-targeted drug (such as TARCEVAO®, IRESSA® or Cetuximab); an

anti-angiogenic agent (especially Bevacizumab sold by Genentech under the trademark AVASTIN™); a tyrosine kinase inhibitor; a COX inhibitor (for instance a COX-1 or COX-2 inhibitor); non-steroidal anti-inflammatory drug, Celecoxib (CELEBREX®); farnesyl transferase inhibitor (for example, Tipifarnib/ZARNESTRA® R115777 available from Johnson and Johnson or Lonafarnib SCH66336 available from Schering-Plough); antibody that binds oncofetal protein CA 125 such as Oregovomab (MoAb B43.13); HER2 vaccine (such as HER2 AutoVac vaccine from Pharmexia, or APC8024 protein vaccine from Dendreon, or HER2 peptide vaccine from GSK/Corixa); another HER targeting therapy (e.g. trastuzumab, cetuximab, gefitinib, erlotinib, C11033, GW2016 etc); Raf and/or ras inhibitor (see, for example, WO 2003/86467); doxorubicin HCl liposome injection (DOXIL®); topoisomerase I inhibitor such as topotecan; taxane; HER2 and EGFR dual tyrosine kinase inhibitor such as lapatinib/GW572016; TLK286 (TELCYTA®); EMD-7200; a medicament that treats nausea such as a serotonin antagonist, steroid, or benzodiazepine; a medicament that prevents or treats skin rash or standard acne therapies, including topical or oral antibiotic; a body temperature-reducing medicament such as acetaminophen, diphenhydramine, or meperidine; hematopoietic growth factor, etc.

Suitable dosages for any of the above coadministered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and HER antibody.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

Preferably, the antibody administered is a naked antibody. However, the antibody administered may be conjugated with a cytotoxic agent. Preferably, the immunoconjugate and/or antigen to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate in killing the cancer cell to which it binds. In a preferred embodiment, the cytotoxic agent targets or interferes with nucleic acid in the cancer cell. Examples of such cytotoxic agents include maytansinoids, calicheamicins, ribonucleases and DNA endonucleases.

Aside from administration of the antibody protein to the patient, the present application contemplates administration of an antibody or protein inhibitor by gene therapy. See, for example, WO96/07321 published Mar. 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells; in vivo and ex vivo. For in vivo delivery the nucleic acid is injected directly into the patient, usually at the site where the antibody is required. For ex vivo treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, e.g. U.S. Pat. Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for ex vivo delivery of the gene is a retrovirus.

The currently preferred in vivo nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., *J. Biol. Chem.* 262:4429-4432 (1987); and Wagner et al., *Proc. Natl. Acad. Sci. USA* 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson et al., *Science* 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of cancer or the other disorders described above is provided. The article of manufacture comprises a vial with a fixed dose of the HER antibody contained therein and, optionally, a package insert. The vial may be formed from a variety of materials such as glass or plastic, and may be sealed by a stopper pierceable by a syringe. For example, the vial may be a formal vitrum type I glass vial (e.g. 20 cc vial for a 420 mg fixed dose or 50 cc vial for a 1050 mg fixed dose), with DAIKYO GREY™ fluro-resin laminated stopper, and 20 mm flip top aluminum cap. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes, etc.

In the preferred embodiment, the article of manufacture comprises a vial containing a fixed dose of a HER antibody (e.g. pertuzumab), wherein the fixed dose is approximately 420 mg, approximately 525 mg, approximately 840 mg, or approximately 1050 mg of the HER antibody.

The article of manufacture preferably further comprises a package insert. The package insert may provide instructions to administer the fixed dose to a cancer patient, including but not limited to a patient with ovarian cancer, peritoneal cancer, fallopian tube cancer, metastatic breast cancer (MBC), non-small cell lung cancer (NSCLC), prostate cancer, colorectal cancer, and/or to administer the fixed dose to a cancer patient whose cancer displays HER expression, amplification and/or phosphorylation.

In one embodiment, the article of manufacture comprises two vials, wherein a first vial contains a fixed dose of approximately 840 mg of pertuzumab, and a second vial contains a fixed dose of approximately 420 mg of pertuzumab.

In another embodiment, the article of manufacture of comprises two vials, wherein a first vial contains a fixed dose of approximately 1050 mg of pertuzumab, and a second vial contains a fixed dose of approximately 525 mg of pertuzumab.

VII. Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209, USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1

The present example evaluates the population pharmacokinetic (PK) and predictive covariates for the HER antibody pertuzumab, and examined the variability of steady-state trough serum concentrations after fixed, body weight-based, or BSA-based dosing methods. Pertuzumab was administered by IV infusion (q3 week) either as a weight-based dose (0.5-15 mg/kg) or a fixed dose (420 mg or 1050 mg). Pertuzumab serum concentration data from one phase Ia and two phase II trials (ovarian and breast), comprising 153 patients and 1458 concentration-time points, were pooled for this analysis using NONMEM™ with the first-order conditional estimation with Interaction (FOCE interaction) method. A linear 2-compartment model best described the data. Body weight, serum albumin, and serum alkaline phosphatase were significant covariates affecting clearance (CL), and body-surface area (BSA) was a significant variable affecting distribution volume at central compartment (Vc). In the final model, CL and Vc were 0.214 L/day and 2.74 L, respectively. Weight only explained 8.3% of inter-patient variability for CL. Evaluation of the final population PK model using a posterior predictive check showed good performance. Compared to fixed dosing, weight- and BSA-based dosing only reduced the population variability of steady-state trough concentrations by 6.2% and 5.8%, respectively, in 1000 simulated subjects bootstrapped from the original data set using the final model. Simulations also showed that the percentages of subjects with predicted steady-state trough concentrations below a target of 20 mcg/mL were similar following fixed, weight- or BSA-based dosing. It was concluded that although humanized antibodies are typically dosed by weight, the analyses in this example demonstrate the desirability of administering the HER antibody, pertuzumab, using a fixed dose to treat cancer.

Methods

Studies and Patients

All three studies used in this analysis were approved by the appropriate ethics committees of the participating centers. Written informed consent was obtained from all patients.

Study 1 was a Phase Ia, open-label, multicenter, dose-escalation study to evaluate the safety, tolerability, and pharmacokinetic profiles of pertuzumab administered intravenously as a single agent to subjects with advanced solid malignancies. These patients received a dose of pertuzumab administered by the IV route every 3 weeks as a 90-minute IV infusion on the 1st cycle, then as 30-minute infusion in subsequent cycles. Doses were escalated (0.5, 2, 5, 10, and 15 mg/kg) in cohorts of 3 or 6 subjects until the maximum tolerated dose (MTD) was defined or the highest dose level was reached. During the first cycle of treatment, serum samples for determination of pertuzumab concentrations were collected at serial time points: prior to the dose, at the end of the IV infusion, at 1.5, 4, and 9 hours, and on days 2, 5, 8, and 15. During the second treatment cycle, serum samples for determination of pertuzumab concentrations were collected prior to the dose, 29 minutes following the start of the IV infusion, and on day 8.

Study 2 was a phase II, open-label, single-arm, multicenter trial to evaluate the overall efficacy, safety, tolerability and the effect of tumor-based HER2 activation on the efficacy of pertuzumab in patients with advanced ovarian cancer, in which their disease was refractory to or had recurred following prior chemotherapy. These women received IV infusions of pertuzumab administered as a single agent over a 90-minute period during the 1st cycle of treatment at a fixed dose of 840 mg, followed by a 420 mg maintenance dose, delivered as a 30-minute infusion every 3 weeks during subsequent treatment cycles. During the first and second treatment cycle, serum samples for determination of pertuzumab concentrations were collected prior to the dose, 15 minutes following the end of the infusion, and at days 8 and 15. Additional serum samples for determination of pertuzumab concentrations were collected prior to the dose and 15 minutes after the end of the IV infusion during subsequent treatment cycles.

Study 3 was a Phase II, open label, single-arm, multicenter randomized study to evaluate the efficacy and safety of two different doses of pertuzumab administered as a single agent in patients with metastatic breast cancer with low expression of HER2. In first dose cohort, patients received pertuzumab as an IV infusion administered over a 90 minute period as a 840 mg loading dose on the 1st cycle, followed by a maintenance dose of 420 mg given every 3 weeks as a 30 minute IV infusion during subsequent treatment cycles. In the second dose cohort, the patients received an IV infusion of pertuzumab as a 1050 mg dose over a 90-minute period on the 1st cycle, and as a 1050 mg dose as a 30-minute IV infusion every 3 weeks during subsequent treatment cycles. In study 3, serum samples for determination of pertuzumab concentrations were collected prior to the dose, 15 minutes following the end of infusion, and on days 8 and 15 during the first two treatment cycles. Additional serum samples for determination of pertuzumab concentrations were collected prior to the dose and 15 minutes after the end of infusion during subsequent treatment cycles.

Drug Assay

Pertuzumab serum concentrations were determined by a validated receptor-binding, enzyme -linked, immunosorbent assay (ELISA). The assay used p185HER2 extracellular domain to capture pertuzumab from serum samples. Bound pertuzumab was detected with mouse anti-human Fc-horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, Inc.), and tetramethyl benzidine (TMB) (KPL, Inc.) was used as the substrate for color development to quantify serum pertuzumab. The assay has a minimum quantifiable concentration of 0.25 mcg/mL for pertuzumab in human serum.

Population Pharmacokinetic Analysis

Population non-linear mixed-effect modeling was performed using NONMEM™ (Boeckmann and Beal NONMEM™ User Guide. San Francisco: NONMEM™ Project Group, University of California, San Francisco (1994)) software (Version V, Level 1.0) with NM-TRAN and PREDPP and the Compaq Visual Fortran compiler (Version 6.5). Two different basic structural models, a one- and two-compart-

mental linear PK model with IV infusion, were fit to serum pertuzumab concentration-time data. The first-order conditional estimation (FOCE) method with η-ϵ interaction was used throughout the model-building procedure. An exponential error model was used to describe the interindividual variability for the PK parameters:

$$Pi=\hat{P}_i \exp(\eta_{iP}) \quad (1)$$

A multiplicative covariate regression model was implemented as follows:

$$\hat{P}i = \theta_1\left(\frac{X_i}{med\,(X)}\right)^{\theta x}(1+\theta_D D) \quad (2)$$

where η_{ip} denotes the proportional difference between the "true" parameters (Pi) of i^{th} individual patients and the typical value ($\hat{P}_i$) in the population, adjusted for values of covariates equal to those of this individual patient. The η_{ip} is the random effects with mean zero and variance ω^2. The θ_X, and θ_D are the regression coefficients to be estimated for continuous (e.g. WT) or dichotomous (e.g. SEX and RACE) covariates, respectively. Continuous variables were centered on their median (med (X)) values, thus allowing θ_1 to represent the clearance estimate for the typical patient with median covariates. Dichotomous covariates were coded 0 or 1 (e.g., SEX=0 for female, SEX=1 for male; RACE=0 for Caucasian, and RACE=1 for others). The residual variability was modeled as proportional-additive error model:

$C_{pij}=\hat{C}_{pij}\,(1+\epsilon_{ij,prop})+\epsilon_{ij,add}$, where C_{pij} and $\hat{C}_{pij}$ are the j^{th} measured and model-predicted concentration, respectively, for i^{th} individual, and $\epsilon_{ij,prop}$ and $\epsilon_{ij,add}$ denote the proportional and additive residual intra-individual random errors distributed with zero means and variances σ_{prop}^2 and σ_{add}^2.

The relationships between structural model-based Bayesian estimates of the PK parameters and individual covariates were explored graphically. Based on preliminary exploratory analyses, the effect of each covariate on PK parameters was tested. Initially, the influence of covariates on individual PK parameters (i.e., clearance and volume of the central compartment) was examined. Then, a full covariate model for the individual PK parameters was constructed by incorporating the significant covariates into the model. A backward elimination process was used to determine the final covariate model for each individual PK parameter by retaining only the significant covariates in the model. When highly correlated covariates had a similar pharmacological meaning (such as weight and BSA), only the most significant factor was retained in the model. This final covariate model obtained for each PK parameter was then combined to form a new full model and the final population PK model was elaborated using a backward elimination process.

Comparison of alternative structural models and construction of the covariate model was based on the typical goodness-of-fit diagnostic plots and likelihood ratio test. When comparing alternative hierarchical models, the differences in the value of the objective function is approximately chi-square distributed with n degree of freedom (n is the difference in the number or parameters between the full and the reduced model). This approximation has been shown to be reliable for the FOCE-INTERACTION estimation method (Wahlby et al. *J Pharmacokinet Pharmacodyn* 28:231-52 (2001)). To discriminate two hierarchical models, a difference in an objective function of greater than 7.9 (1 degree of freedom), which corresponds to a significance level of $p<0.005$, was used.

The fraction of inter-individual variance (% variance) explained by the covariates in the regression model for a given PK parameters (e.g., CL) was computed as follows:

$$\%\ \text{variance} = \left(\frac{\omega^2_{CL,BASED}-\omega^2_{CL,FINAL}}{\omega^2_{CL,BASE}}\right)\times 100 \quad (3)$$

Where $\omega^2_{CL,BASED}$ and $\omega^2_{CL,FINAL}$ represented inter-individual variance of clearance in based and final PK model, respectively.

Population Pharmacokinetic Model Evaluation

The model evaluation in this study utilized a bootstrap resampling technique to evaluate the stability of the final model and estimate the confidence interval of parameters. This model evaluation technique consists of first creating data sets using the bootstrap option in the software package Wings for NONMEM™ (N Holford, Version 404, June 2003, Auckland, New Zealand) then obtaining parameter estimates for each of the replicate data sets. The results from 1000 successful runs were obtained, and the mean and $_{2.5}{}^{th}$ and 97.5^{th} percentiles (denoting the 95% confidence interval) for the population parameters were determined and compared with the estimates of the original data.

In addition, a posterior predictive model checks were used to evaluate the ability of the final model to describe the observed data (Yano et al. *J Pharmacokinet Pharmacodyn* 28:171-92 (2001); Gelman and Meng, Model checking and model improvement. In: Gilks W R, Richardson S, Spiegelhalter D J, eds. *Markov Chain Monte Carlo in Practice*. Boca Raton: Chapman & HalVCRC, 189-202 (1996); Gelman et al. *Bayesian Data Analysis*. Boca Raton: Chapman & Hall/CRC (2004). In these analyses, the 2.5^{th}, 5^{th}, 10^{th}, 25^{th}, 50^{th} (median), 75^{th}, 90^{th}, and 95^{th} percentiles of the observed data were computed and selected as the test statistics for the posterior predictive model check. The final population PK model, including final fixed and random-effect parameters, was used to simulate 1000 replicates of the observed data set and test statistics were computed from each of those simulated dataset. The posterior predictive distribution of test statistics from the simulated dataset was then compared with the observed test statistics, and the p-value (p^{PPC}) can be estimated by calculating the proportion of cases in which test statistics from the simulated data set exceed the realized value of observed test statistics according to the following equation (Gelman and Meng, (1996) supra).

$$p^{PPC} = \frac{1}{N}\sum_{i=1}^{1000} I\,(T\,(y_i^{rep},\theta) \geq T\,(y,\theta_i)) \quad (1)$$

Where I(.) is the indicator function which takes the value 1 when its argument is true and 0 otherwise. T(y,θ) is a 'realized value' of the observed test statistics because it is realized by the observed data y. $T(y_i^{rep},\theta)$ is the test statistics from a simulated data set i (range from 1 to 1000) (Gelman and Meng, (1996) supra).

In addition, the 2.5^{th}, 5^{th}, 95^{th}, and 97.5^{th} quantiles of the simulated data were calculated for each time points for individual patients. The numbers of observed data that fell within the boundaries of the 2.5^{th} and 95.5^{th} quantiles (95% interval), 5^{th} and 95^{th} quantiles (90% interval) of the pooled simulated data were determined.

Pertuzumab Exposures after Fixed, BSA-, and Weight-Based Dosing

The final population PK model was used to determine the steady state trough concentrations and exposure after fixed,

BSA-, and weight-based dosing. Serum concentration-time profiles and clearance of pertuzumab for 1000 subjects were simulated for a fixed, BSA-based, or weight-based dosing regimen using the final model with a dataset obtained by bootstrapping (with replacement) the original PK dataset. All simulated subjects received a 840 mg, 12.2 mg/kg, or 485 mg/m2 iv infusion over 90 min on Day 0, then a 420 mg, 6.1 mg/kg, or 242.5 mg/m iv infusion over 30 min on Days 21, 42 and 63. Steady state trough concentrations obtained on Day 84 (Css_{trough}) after different dosing regimens were then assessed. In addition, the percent of subjects with steady state trough concentrations below a target concentration (20 mcg/ml) after a fixed, BSA-based or weight-based dose were calculated. Simulated clearance values were used to determine the steady state average exposure ($AUCss_{0-\tau}$) according to the following equation:

$$AUC_{SS0-\tau} = \frac{\text{Dose}}{CL} \quad (4)$$

Demographic data. The demographic characteristics of the patients included in this PK analysis are listed in Table 2.

TABLE 2

Demographic characteristics of the patients included in the analysis

	Median	Range
Age (years)	56.0	32.0-78.0
BSA* (m^2)	1.73	1.40-2.53
Weight (kg)	69.0	45.0-150.6
Albumin (g/L)	39.2	21.0-52.0
Alkaline phosphatase (ALK) (IU/L)	107.0	39.0-367.0
	Number of Patients	**%**
Gender		
Male	8	5.2
Female	145	94.8
Race		
Caucasian	141	92.2
African American	3	2.0
Hispanic	4	2.6
Asian	3	2.0
Native Indian	0	0
Others	2	1.3

*BSA, body surface area.

A total of 1458 pertuzumab serum concentration time points were collected from 153 patients in the three studies. Of the total, 18 patients were from the phase Ia trial, 60 from the phase II ovarian cancer trial, and 75 from the phase II breast cancer trial. Thus, the majority (94.8%) of the patients in this analysis were female and accounted for 1110 (76%) of the serum pertuzumab concentration data. All subjects had low HER2 expression tumor confirmed by FISH (fluorescence in situ hybridization) analysis and had good physical functional status as indicated by an ECOG (Eastern Cooperative Oncology Group) performance status of either 0 or 1. The number of patients with missing covariates was very low (4.6% for both height and BSA) and the missing covariates were imputed with the median values. In 384 (20.8%) serum pertuzumab concentration samples with only a documented sampling date, the sampling time was imputed to occur at 12 noon. A sensitivity analysis conducted to assess the effects of these imputed times on the population parameters estimates in the model revealed no significant influence.

Population PK analysis. A two-compartment model described the data better than one-compartment model based on the change of objective function (δ=−736.2) and diagnostic plots. A representative pertuzumab serum concentration-time profile fit to a one- and two-compartment model are illustrated in FIGS. **9**A-B. The inter-individual variability term (η) of K12 was removed from two-compartmental models since the removal of this η term did not result in a statistically significant increase (δ<7.88) in the objective function. Therefore, only η_{CL}, η_{Vc} and η_{k21} were retained in the final base model.

The effect of the presence of a covariance term among η_{CL}, η_{Vc} and η_{k21} was next assessed. Incorporation of a covariance terms among η_{CL}, η_{Vc} and η_{k21} improved the fit (δ=−23.2, df=3). However, the covariance terms were found to be poorly estimated (% CV>100), have a small estimated correlation ($r_{CL\text{-}Vc}$=0.37; $r_{CL\text{-}K21}$=0.27; $r_{Vc\text{-}K21}$=0.42), and have little influence on parameter estimations (data not shown). Therefore, the covariance terms were not retained for covariate effect model building. In an exploratory analysis using the final base model, no apparent relationships between potential covariates and η_{k21} were identified. Therefore, covariate effect on η_{k21} was not examined during the development of the final model with covariates.

For the final model with covariates, predicted versus observed pertuzumab serum concentrations and weighted residuals versus predicted serum concentration plots are shown in FIGS. **10**A-B. In the final model, serum albumin (ALB), body weight (BW) and serum alkaline phosphatase (ALKP) were the most significant covariates explaining inter-individual variability for pertuzumab clearance (CL). BSA was the most significant covariate explaining interindividual variability of pertuzumab central compartment volume of distribution (Vc). Incorporation of covariance terms among η_{CL}, η_{Vc} and η_{k21} improved the fit (δ=−14.0,df=3). However, the estimated correlation was not large ($r_{CL\text{-}Vc}$=0.45; $r_{CL\text{-}K21}$=0.28; $r_{Vc\text{-}K21}$=0.39) and the parameter estimates were not influenced (data not shown). Hence, the covariance terms were not included in the final model. The final model was illustrated as follows:

$$CL = \theta_{CL} \times \left(\frac{WT}{69}\right)^{\theta_{WT\_CL}} \times \left(\frac{ALB}{39.2}\right)^{\theta_{ALB\_CL}} \times \left(\frac{ALKP}{107}\right)^{\theta_{ALKP\_CL}} \quad (4)$$

$$Vc = \theta_{Vc} \times \left(\frac{BSA}{1.72}\right)^{\theta_{BSA\_Vc}} \quad (5)$$

The parameter estimates of the final model are summarized in Table 3.

TABLE 3

Parameter Estimates of the Final Population Pharmacokinetic Model and the Stability of the Parameters Using a Bootstrap Validation Procedure

	Original Data Set Estimate (% RSE)[a]	1000 bootstrap Replicates Mean (95% CI)
Structural Model		
CL (L/day)	0.214 (3.1)	0.214 (0.201, 0.228)
Vc (L)	2.740 (1.9)	2.739 (2.640, 2.840)
K12 (day^{-1})	0.203 (16.6)	0.220 (0.159, 0.416)
K21 (day^{-1})	0.258 (15.6)	0.275 (0.203, 0.480)

TABLE 3-continued

Parameter Estimates of the Final Population Pharmacokinetic Model and the Stability of the Parameters Using a Bootstrap Validation Procedure

	Original Data Set Estimate (% RSE)[a]	1000 bootstrap Replicates Mean (95% CI)
Inter-individual Variability		
CL % CV	31.1 (11.0)	30.6 (27.0, 34.1)
Vc % CV	16.2 (20.3)	16.0 (12.7, 19.2)
K_{21} % CV	25.2 (37.6)	24.1 (11.4, 33.6)
Covariate Model		
ALB on CL (θ_{ALB_CL})	−1.010 (18.4)	−1.019 (−1.420, −0.632)
WT on CL (θ_{WT_CL})	0.587 (19.3)	0.589 (0.372, 0.826)
ALKP on CL (θ_{ALKP_Vc})	0.169 (29.5)	0.170 (0.067, 0.258)
BSA on Vc (θ_{BSA_Vc})	1.160 (12.2)	1.151 (0.890, 1.451)
Residual Variability		
Proportional error σ^2_{prop}	0.037 (19.4)	0.037 (0.030, 0.045)
Additive error, σ_{prop}, mcg/mL	2.265 (77.8)	2.24 (0.002, 4.160)

[a]% RSE: percent relative standard error of the estimate = SE/parameter estimate × 100

The CL of serum pertuzumab in the analysis population was estimated to be 0.214 L/day and the Vc was 2.74 L. The K_{12} and K_{21} were 0.203 and 0.258 $days^{-1}$, respectively. Inter-individual variability for CL and Vc in the final model, calculated as the square root of interindividual variance (ω^2) and expressed as % CV, are 31.1% and 16.2%, respectively, compared to 38.0% and 20.8% for the base model without covariates. The covariate effect of ALB, WT, and ALKP in the final model therefore explained about 33% of the interindividual variance for CL. However, weight alone explained only 8.3% of inter-patient variability for CL. The covariate effect of BSA explained about 39% of interindividual variance for Vc in the final model. The dependency of CL on WT and Vc on BSA with the base model is accounted for in the final model as shown in FIGS. **11**A-B. The estimated $t_{1/2\alpha}$ and $t_{1/2\beta}$ were 1.4 and 17.2 days, respectively.

Model Evaluation. From the original dataset, 1000 successful bootstrap runs were obtained and compared to the original observed data. Mean population PK estimates obtained from the bootstrap procedure were similar to the parameter estimates of the original dataset (Table 3), indicating that the developed model was stable. The 95% confidence intervals for the fixed-effect parameters were narrow, which indicated good precision.

A posterior predictive model check was used to evaluate the ability of the final model to describe the observed data. The final population pharmacokinetic model, including final fixed and random -effect parameters, was used to simulate 1000 replicates. The test statistics were then computed for each of those 1000 simulated dataset. FIGS. **12**A-F display histograms of the 1000 simulated values of selected test statistics, with the "realized value" of the observed test statistics indicated by vertical line. The posterior predictive distributions were close to the observed values with the estimated p-values greater than 0.05 for each test statistics. In addition, the percents of observed pertuzumab concentrations within 90% and 95% quantile range of the pooled simulated data were 89.3 and 94.7%, respectively. These results suggested that the model was able to describe and predict the data reasonably well.

Pertuzumab Exposures after Fixed, BSA-, and Weight-Based Dosing.

Predicted pertuzumab steady-state trough serum concentrations on day 84 ($C_{ss,trough}$) were estimated for 1000 simulated subjects bootstrapped from the original PK dataset and the final model using a fixed, weight-based, or BSA-based dose according to the dose schedules outlined in the methods section. These data showed that with weight-based and BSA-based dosing, population variability of $C_{ss,trough}$ decreased by 6.17 and 5.76%, respectively, when compared to fixed dosing (FIG. **13** and Table 4).

TABLE 4

Predicted Pertuzumab Steady State Trough Concentration (Day 84) After a Fixed, Weight or BSA-based Dose for 1000 Simulated Subjects Bootstrapped from Original PK Dataset According to the Final Model

	Css trough (mcg/ml) Fixed Dose	Css trough (mcg/ml) Weight-based Dose	Css trough (mcg/ml) BSA-based Dose
Minimum	2.68	2.39	2.54
5th Percentile	16.56	16.32	16.86
Median	51.87	51.81	52.48
Mean	56.37	56.08	56.44
95th Percentile	115.38	110.46	112.14
Maximum	209.67	179.06	192.00
% CV	54.05	52.62	52.40
Variance	928.21	870.93	874.71
% Variance Changed from Fixed Dose[a]	—	−6.17	−5.76
Percent of Subjects with $C_{sstrough} \leq 20$ mcg/ml	8.3	8.7	8.3

[a]Percent variance changed from fixed dose was calculated using the following equation:

$$\text{Percent variance change} = \frac{\text{Variance WT or BSA – based dose} - \text{Variance fixed dose}}{\text{Variance fixed dose}} \times 100$$

The percentage of subjects with $C_{sstough}$ below a target serum concentration of 20 mcg/ml were similar, with values of 8.3%, 8.7%, and 8.3% for fixed, weight-based, or BSA -based dosing, respectively (Table 4). Similar results were obtained from the analysis of pertuzumab serum steady state $AUC_{ss_{0-\tau}}$ for 1000 simulated subjects, and weight- and BSA-based dosing only reduced the population variability by 2.2 and 4.2%, respectively, when compared to fixed dosing. The same simulated dataset was used to determine $C_{ss,trough}$ after a fixed dose, weight-, and BSA-based dose for populations with extreme weight (i.e., WT≦10^{th} and ≧90^{th} percentile) (FIGS. **14**A-B). Median pertuzumab $C_{ss,trough}$ for population with WT less than or equal to 10^{th} percentile were 72.3 (range: 8.7 to 166.5), 52.8 (range: 6.8 to 125.7) and 63.2 (range: 7.8 to 150.1) mcg/ml for a fixed dose, weight-, and BSA-based dose, respectively. The percentage of subjects in population with $C_{ss,tough}$ below a target serum concentration of 20 mcg/ml were 5.4%, 12.6%, and 9.0% for fixed, weight-based, and BSA-based dosing, respectively. Median pertuzumab $C_{ss,trough}$ for population with WT greater than or equal to 90^{th} percentile were 42.1 (range: 7.0 to 119.8), 62.8 (range: 14.4 to 167.3) and 52.9 (range: 10.2 to 133.3) mcg/ml for a fixed

dose, weight-, and BSA-based dose, respectively. The percentages of subjects in this population with $C_{ss,trough}$ below a target serum concentration of 20 mcg/ml were similar, with values of 7.4%, 2.8%, and 5.6% for fixed, weight-based, or BSA-based dosing, respectively. Similar results were obtained for the analysis of pertuzumab serum steady state $AUC_{ss_{0-\tau}}$ of these subgroups from 1000 simulated subjects.

DISCUSSION

Typically, humanized IgG monoclonal antibodies and cytotoxic small molecule drugs in oncology have been administered on a weight-based (mg/kg) or BSA-based dose basis. Pertuzumab has undergone testing in the clinic with a phase Ia trial in patients with advanced cancers and in phase II trials in patients with ovarian, breast, lung, and prostate cancer. Pertuzumab was dosed on a weight-basis (mg/kg) in a Phase I trial, and then initiated using a fixed dose in a phase II trials. Using demographic and serum pertuzumab concentration-time data collected in these three trials, a population PK model with predictive covariates for pertuzumab PK was built herein. This model was then used to examine the steady-state concentrations after fixed dosing, weight- and BSA-based dosing methods.

Pertuzumab PK obtained from this analysis was very similar to those reported for other humanized monoclonal IgG1 drugs used in oncology (Harris et al. *Proc Am Soc Clin Oncol* 21:488a (2002); Leyland-Jones et al. *J Clin Oncol* 21:3965-71 (2003); and Lu et al. *Clin Pharmacol Ther* 75:91 (2004)).

A linear 2-compartment linear PK model best describe the data and in the final model pertuzumab CL was 0.214 L/day. Typical Vc of pertuzumab was 2.74 L or approximately 40 ml/kg, which is equal to human plasma volume and was consistent with values reported for other monoclonal IgG1 drugs (Harris et al. (2002), supra; and Lu et al. (2004), supra). Pertuzumab CL was significantly affected by body weight and serum concentrations of albumin and alkaline phosphatase, while Vc was significantly influenced by BSA. The effect of gender on the pertuzumab PK cannot be assessed because of the small number of male subjects (5.2%) included in the analysis. The results from a bootstrap procedure and posterior model checking suggested that the final model was stable and able to describe and predict the data reasonably well.

The effect of weight on CL and BSA on Vc suggested that pertuzumab might be dosed based on either body weight or BSA. However, the covariate effect of weight alone and BSA alone in the model only explained about 8.3% and 40% of the inter-individual effect of CL and Vc, respectively. This suggested that while weight is a predictor of CL and BSA is a predictor for Vc, the effect of weight and BSA on pertuzumab exposures after dosing might be measurable but not highly contributory.

Therefore, the next step assessed the impact of the various dosing methods on the pertuzumab exposures using simulations. In 1000 subjects bootstrapped from the original data set, weight-based or BSA-based dosing were found to decrease population variability of simulated steady state trough serum concentrations on day 84 by only 6.2 and 5.8%, respectively, when compared to fixed dosing. In addition, the percentages of subjects with predicted steady-state trough serum concentrations below a selected target of 20 mcg/mL were similar with all three dosing methods. Similar results were obtained from the subgroup analysis in population with extreme body weight (i.e., WT$\leqq 10^{th}$ and $\geqq 90^{th}$ percentile).

Hence, it is concluded that pertuzumab PK is related to WT and BSA. However, the WT and BSA explained only a small percent of the inter-individual variability of CL and Vc, and WT- and BSA -based dosing do not seem to improve the predictability of pertuzumab steady state exposures. It is recommended to apply fixed-dosing regimens for pertuzumab in cancer patients.

The present invention is believed to represent the first disclosure of a critical assessment of the impact of weight- or BSA-based dosing of a humanized IgG1 monoclonal antibody on steady state drug concentrations in cancer patients. Implementation of flat-fixed dosing has several significant patient care and economic implications: i) lower costs due to greater efficiency in manufacturing, storing and shipping of single unit dose, ii) efficient preparation of a single dose in pharmacies and hospitals without the need for patient individualization, iii) greater efficiency in physician prescribing of single unit dose, and iv) lower likelihood of patient receiving wrong dose due to dose calculation errors. Although humanized antibodies are typically dosed by weight or BSA, the analyses herein demonstrate the feasibility of administrating the HER antibody pertuzumab using a fixed dose in cancer patients.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 22

<210> SEQ ID NO 1
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 1

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Asp Thr Val Met Thr Gln Ser His Lys Ile Met Ser Thr Ser Val
  1               5                  10                  15

Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Ser
                20                  25                  30

Ile Gly Val Ala Trp Tyr Gln Gln Arg Pro Gly Gln Ser Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Asp
                50                  55                  60
```

-continued

```
Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
                65                  70                  75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu
                95                 100                 105

Ile Lys


<210> SEQ ID NO 2
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 2

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
  1               5                  10                  15

Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Thr Phe Thr
                20                  25                  30

Asp Tyr Thr Met Asp Trp Val Lys Gln Ser His Gly Lys Ser Leu
                35                  40                  45

Glu Trp Ile Gly Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                50                  55                  60

Asn Gln Arg Phe Lys Gly Lys Ala Ser Leu Thr Val Asp Arg Ser
                65                  70                  75

Ser Arg Ile Val Tyr Met Glu Leu Arg Ser Leu Thr Phe Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
               110                 115


<210> SEQ ID NO 3
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence is synthesized

<400> SEQUENCE: 3

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser
                20                  25                  30

Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys


<210> SEQ ID NO 4
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<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence is synthesized

<400> SEQUENCE: 4

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1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
20 25 30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
50 55 60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
95 100 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
110 115

<210> SEQ ID NO 5
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence is synthesized

<400> SEQUENCE: 5

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
20 25 30

Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys

<210> SEQ ID NO 6
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence is synthesized

<400> SEQUENCE: 6

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser

-continued

```
                20                  25                  30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
                95                 100                 105

Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
               110                 115


<210> SEQ ID NO 7
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: 10
<223> OTHER INFORMATION: Xaa is preferrably D or S

<400> SEQUENCE: 7

Gly Phe Thr Phe Thr Asp Tyr Thr Met Xaa
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<210> SEQ ID NO 8
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 8

Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr Asn Gln Arg Phe
  1               5                  10                  15

Lys Gly


<210> SEQ ID NO 9
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 9

Asn Leu Gly Pro Ser Phe Tyr Phe Asp Tyr
  1               5                  10


<210> SEQ ID NO 10
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 10

Lys Ala Ser Gln Asp Val Ser Ile Gly Val Ala
  1               5                  10
```

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<210> SEQ ID NO 11
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence is synthesized
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: 5
<223> OTHER INFORMATION: Xaa is preferably R or L
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: 6
<223> OTHER INFORMATION: Xaa is preferably Y or E
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: 7
<223> OTHER INFORMATION: Xaa is preferably T or S

<400> SEQUENCE: 11

Ser Ala Ser Tyr Xaa Xaa Xaa
1 5

<210> SEQ ID NO 12
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 12

Gln Gln Tyr Tyr Ile Tyr Pro Tyr Thr
1 5

<210> SEQ ID NO 13
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 13

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser
20 25 30

Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
110 115 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
125 130 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
140 145 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
155 160 165

-continued

```
Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys
```

<210> SEQ ID NO 14
<211> LENGTH: 448
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 14

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
                20                  25                  30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
                110                 115                 120

Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
                125                 130                 135

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp
                140                 145                 150

Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu
                155                 160                 165

Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly
                170                 175                 180

Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
                185                 190                 195

Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn
                200                 205                 210

Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr
                215                 220                 225

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
                230                 235                 240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
                245                 250                 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
                260                 265                 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
                275                 280                 285
```

-continued

```
Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
                290                 295                 300

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp
                305                 310                 315

Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
                320                 325                 330

Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
                335                 340                 345

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met
                350                 355                 360

Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
                365                 370                 375

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
                380                 385                 390

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
                395                 400                 405

Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
                410                 415                 420

Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
                425                 430                 435

Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                440                 445


<210> SEQ ID NO 15
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 15

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                 20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180
```

-continued

```
Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys
```

<210> SEQ ID NO 16
<211> LENGTH: 449
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence is synthesized

<400> SEQUENCE: 16

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                 20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                 50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                 65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                 95                 100                 105

Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                 120

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser
                125                 130                 135

Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys
                140                 145                 150

Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
                155                 160                 165

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
                170                 175                 180

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
                185                 190                 195

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
                200                 205                 210

Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
                215                 220                 225

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                230                 235                 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
                260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
                275                 280                 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
                290                 295                 300

Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
```

-continued

```
                305                 310                 315

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
                320                 325                 330

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
                335                 340                 345

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
                350                 355                 360

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
                365                 370                 375

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
                380                 385                 390

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
                395                 400                 405

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
                410                 415                 420

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
                425                 430                 435

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                440                 445


<210> SEQ ID NO 17
<211> LENGTH: 217
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 17

Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
  1               5                  10                  15

Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln
                 20                  25                  30

Asp Val Ser Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys
                 35                  40                  45

Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly
                 50                  55                  60

Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
                 65                  70                  75

Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr
                 80                  85                  90

Cys Gln Gln Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr
                 95                 100                 105

Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile
                110                 115                 120

Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val
                125                 130                 135

Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln
                140                 145                 150

Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser
                155                 160                 165

Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
                170                 175                 180

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
                185                 190                 195

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys
```

-continued

```
                200                 205                 210

Ser Phe Asn Arg Gly Glu Cys
                215


<210> SEQ ID NO 18
<211> LENGTH: 449
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 18

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
                 20                  25                  30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                 50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
                 65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                 95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
                110                 115                 120

Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
                125                 130                 135

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp
                140                 145                 150

Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu
                155                 160                 165

Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly
                170                 175                 180

Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
                185                 190                 195

Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn
                200                 205                 210

Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr
                215                 220                 225

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
                230                 235                 240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
                245                 250                 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
                260                 265                 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
                275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
                290                 295                 300

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp
                305                 310                 315

Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
```

-continued

```
                320                 325                 330

Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
                335                 340                 345

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met
                350                 355                 360

Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
                365                 370                 375

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
                380                 385                 390

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
                395                 400                 405

Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
                410                 415                 420

Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
                425                 430                 435

Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
                440                 445


<210> SEQ ID NO 19
<211> LENGTH: 195
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 19

Thr Gln Val Cys Thr Gly Thr Asp Met Lys Leu Arg Leu Pro Ala
  1               5                  10                  15

Ser Pro Glu Thr His Leu Asp Met Leu Arg His Leu Tyr Gln Gly
                 20                  25                  30

Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr Leu Pro Thr
                 35                  40                  45

Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly
                 50                  55                  60

Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln
                 65                  70                  75

Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
                 80                  85                  90

Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr
                 95                 100                 105

Pro Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu
                110                 115                 120

Arg Ser Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg
                125                 130                 135

Asn Pro Gln Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile
                140                 145                 150

Phe His Lys Asn Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn
                155                 160                 165

Arg Ser Arg Ala Cys His Pro Cys Ser Pro Met Cys Lys Gly Ser
                170                 175                 180

Arg Cys Trp Gly Glu Ser Ser Glu Asp Cys Gln Ser Leu Thr Arg
                185                 190                 195


<210> SEQ ID NO 20
<211> LENGTH: 124
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
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Ile Cys Glu Leu His Cys Pro Ala Leu Val Thr Tyr Asn Thr Asp
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Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu Ser Thr Asp
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Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile
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Arg Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln
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Gly Ser Gly Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe
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Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
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Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val
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Ala Cys Ala His Tyr Lys Asp Pro Pro Phe Cys Val Ala Arg Cys
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Pro Ser Gly Val Lys Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys
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Phe Pro Asp Glu Glu Gly Ala Cys Gln Pro Cys Pro Ile Asn Cys
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Thr His Ser Cys Val Asp Leu Asp Asp Lys Gly Cys Pro Ala Glu
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Gln Arg Ala Ser Pro Leu Thr
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What is claimed is:

1. A method for treating HER2 expressing cancer comprising administering one or more fixed dose(s) of HER2 antibody to a human patient in an amount effective to treat the cancer, wherein the fixed dose is selected from the group consisting of approximately 420 mg, approximately 525 mg, approximately 840 mg, and approximately 1050 mg of the HER2 antibody, wherein the HER2 antibody comprises the variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4, respectively.

2. The method of claim **1** wherein the HER2 antibody is pertuzumab.

3. The method of claim **1** wherein the fixed dose is 420 mg of the HER2 antibody.

4. The method of claim **1** wherein the fixed dose is 840 mg of the HER2 antibody.

5. The method of claim **1** wherein the fixed dose is 1050 mg of the HER2 antibody.

6. The method of claim **1** wherein the fixed dose is 525 mg of the HER2 antibody.

7. The method of claim **1** wherein a fixed dose of the HER2 antibody is administered to the patient approximately every week, approximately every 2 weeks, approximately every 3 weeks, or approximately every 4 weeks.

8. The method of claim **7** wherein a fixed dose of the HER2 antibody is administered to the patient approximately every 3 weeks.

9. The method of claim **1** comprising administering a loading dose of approximately 840 mg of the HER2 antibody followed by one or more maintenance doses of approximately 420 mg of the HER2 antibody.

10. The method of claim **9** wherein the maintenance doses are administered approximately every 3 weeks.

11. The method of claim **1** comprising administering a loading dose of approximately 1050 mg of the HER2 antibody followed by one or more maintenance doses of approximately 525 mg of the HER2 antibody.

12. The method of claim **11** wherein the maintenance doses are administered approximately every 3 weeks.

13. The method of claim **1** wherein the HER2 antibody is a naked antibody.

14. The method of claim **1** wherein the HER2 antibody is an intact antibody.

15. The method of claim **1** wherein the HER2 antibody is an antibody fragment comprising an antigen binding region.

16. The method of claim **1** wherein the HER2 antibody is a humanized or human IgG1antibody.

17. The method of claim **1** wherein the cancer displays HER2 expression, amplification, or activation.

18. The method of claim **1** wherein the cancer is ovarian, peritoneal, or fallopian tube cancer.

19. The method of claim **1** wherein the cancer is metastatic breast cancer (MBC).

20. The method of claim **1** wherein the cancer is non-small cell lung cancer (NSCLC).

21. The method of claim **1** wherein the cancer is prostate cancer.

22. The method of claim **1** wherein the cancer is colorectal cancer.

23. The method of claim **1** comprising administering a second therapeutic agent to the patient.

24. The method of claim **23** wherein the second therapeutic agent is selected from the group consisting of chemotherapeutic agent, different HER2 antibody, antibody directed against a different tumor associated antigen, anti-hormonal compound, cardioprotectant, cytokine, EGFR-targeted drug, anti-angiogenic agent, tyrosine kinase inhibitor, COX inhibitor, non-steroidal anti-inflammatory drug, farnesyl transferase inhibitor, antibody that binds oncofetal protein CA 125, HER2 vaccine, another HER targeting therapy, Raf or ras inhibitor, doxorubicin HCL liposome injection, topotecan, taxane, dual tyrosine kinase inhibitor, TLK286, EMD-7200, a medicament that treats nausea, a medicament that

prevents or treats skin rash or standard acne therapy, a body temperature-reducing medicament, and a hematopoietic growth factor.

25. The method of claim **24** wherein the second therapeutic agent is a chemotherapeutic agent.

26. The method of claim **25** wherein the chemotherapeutic agent is an antimetabolite chemotherapeutic agent.

27. The method of claim **26** wherein the antimetabolite chemotherapeutic agent is gemcitabine.

28. The method of claim **23** wherein the second therapeutic agent is trastuzumab, erlotinib HCL, or bevacizumab.

29. A method of treating HER2 expressing cancer in a human patient comprising administering at least one fixed dose of pertuzumab to the patient, wherein the fixed dose is selected from the group consisting of approximately 420 mg, approximately 525 mg, approximately 840 mg, and approximately 1050 mg of pertuzumab.

30. The method of claim **29** wherein a fixed dose of pertuzumab is administered to the patient approximately every 3 weeks.

31. The method of claim **29** wherein the cancer is selected from the group consisting of ovarian cancer, peritoneal cancer, fallopian tube cancer, metastatic breast cancer (MBC), non-small cell lung cancer (NSCLC), prostate cancer, and colorectal cancer.

32. The method of claim **29** wherein the fixed dose is selected from the group consisting of 420 mg, 525 mg, 840 mg, and 1050 mg of pertuzumab.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,449,184 B2
APPLICATION NO. : 11/154091
DATED : November 11, 2008
INVENTOR(S) : Allison et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title page,

[*] Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 USC 154(b) by 191 days

Delete the phrase "by 191 days" and insert -- by 648 days --

Signed and Sealed this

Eleventh Day of May, 2010

David J. Kappos

David J. Kappos
Director of the United States Patent and Trademark Office

EXHIBIT N

US008691232B2

(12) **United States Patent**
Derynck et al.

(10) **Patent No.: US 8,691,232 B2**
(45) **Date of Patent: *Apr. 8, 2014**

(54) **EXTENDING TIME TO DISEASE PROGRESSION OR SURVIVAL IN CANCER PATIENTS**

(75) Inventors: **Mika K. Derynck**, San Mateo, CA (US); **Stephen M. Kelsey**, Montara, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/010,985**

(22) Filed: **Jan. 21, 2011**

(65) **Prior Publication Data**

US 2011/0165157 A1 Jul. 7, 2011

Related U.S. Application Data

(63) Continuation of application No. 12/271,564, filed on Nov. 14, 2008, now abandoned, which is a continuation of application No. 11/359,185, filed on Feb. 21, 2006, now abandoned.

(60) Provisional application No. 60/655,277, filed on Feb. 23, 2005.

(51) **Int. Cl.**
A61K 39/395 (2006.01)

(52) **U.S. Cl.**
USPC **424/155.1**; 424/138.1; 424/143.1

(58) **Field of Classification Search**
None
See application file for complete search history.

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Primary Examiner — Laura B Goddard

(74) *Attorney, Agent, or Firm* — Wendy M. Lee

(57) **ABSTRACT**

The present application describes extending time to disease progression or survival in a cancer patient, where the patient's cancer displays HER activation, by treating the patient with a HER dimerization inhibitor, such as pertuzumab.

34 Claims, 18 Drawing Sheets

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* cited by examiner

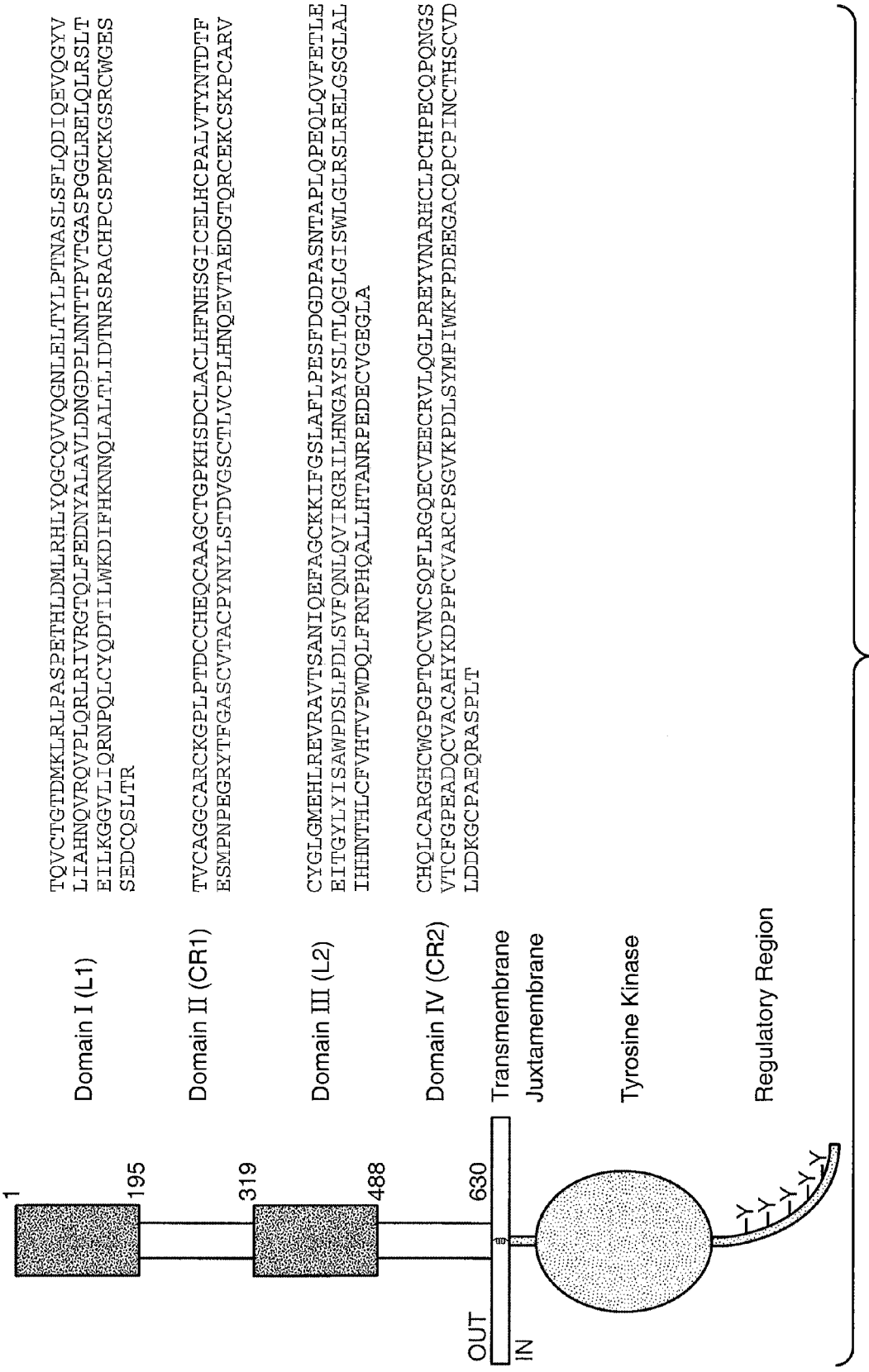

1
195
319
488
630
Domain I (L1)
Domain II (CR1)
Domain III (L2)
Domain IV (CR2)
Transmembrane
Juxtamembrane
Tyrosine Kinase
Regulatory Region
OUT
IN
Y
Y
Y
Y
Y
TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQGYV
LIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLT
EILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGES
SEDCQSLTR
TVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTF
ESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARV
CYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLE
EITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLAL
IHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLA
CHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGS
VTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVD
LDDKGCPAEQRASPLT

FIG. 1

Variable Light

```
                10        20          30          40
2C4       DTVMTQSHKIMSTSVGDRVSITC [KASQDVSIGVA] WYQQRP
           **    **** *      *                     *
574       DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA] WYQQKP
                                   *   ** ***
hum κI    DIQMTQSPSSLSASVGDRVTITC [RASQSISNYLA] WYQQKP

                50          60        70        80
2C4       GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA
           **                  *  *         *    * *
574       GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISSLQP
                   * *****
hum κI    GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP

                90         100
2C4       EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:1)
             * *                 *   *
574       EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:3)
                     *** *
hum κI    EDFATYYC [QQYNSLPWT] FGQGTKVEIK (SEQ ID NO:5)
```

FIG. 2A

Variable Heavy

```
                10        20          30          40
2C4       EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS
              **  **  *  * ***  *                  * *
574       EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA
                                      ** * *
hum III   EVQLVESGGGLVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

                50  a     60          70        80
2C4       HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM
          *  *   **                    *** *    **** *
574       PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL
                    ****** *** ****       * * *
hum III   PGKGLEWVA [VISGDGGSTYYADSVKG] RFTISRDNSKNTLYL

            abc     90         100ab        110
2C4       ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLTVSS (SEQ ID NO:2)
          ***  **                                **
574       QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTLVTVSS (SEQ ID NO:4)
                             ********
hum III   QMNSLRAEDTAVYYCAR [GRVGYSLYDY] WGQGTLVTVSS (SEQ ID NO:6)
```

FIG. 2B

Amino Acid Sequence for Pertuzumab Light Chain

```
1       10        20        30        40        50        60
|       |         |         |         |         |         |
DIQMTQSPSSLSASVGDRVTITC**KASQDVSIGVA**WYQQKPGKAPKLLIYS**ASYRYT**GVPS

        70        80        90        100       110       120
        |         |         |         |         |         |
RFSGSGSGTDFTLTISSLQPEDFATYYC**QQYYIYPYT**FGQGTKVEIKRTVAAPSVFIFPP

        130       140       150       160       170       180
        |         |         |         |         |         |
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT

        190       200       210
        |         |         |
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
```

FIG. 3A

Amino Acid Sequence for Pertuzumab Heavy Chain

```
1       10        20        30        40        50        60
|       |         |         |         |         |         |
EVQLVESGGGLVQPGGSLRLSCAAS**GFTFTDYTMD**WVRQAPGKGLEWVA**DVNPNSGGSIY**

        70        80        90        100       110       120
        |         |         |         |         |         |
**NQRFK**GRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARN**LGPSFYFDY**WGQGTLVTVSSA

        130       140       150       160       170       180
        |         |         |         |         |         |
STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG

        190       200       210       220       230       240
        |         |         |         |         |         |
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGP

        250       260       270       280       290       300
        |         |         |         |         |         |  *|
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS

        310       320       330       340       350       360
        |         |         |         |         |         |
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM

        370       380       390       400       410       420
        |         |         |         |         |         |
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ

        430       440       448
        |         |         |
QGNVFSCSVMHEALHNHYTQKSLSLSPG
```

FIG. 3B

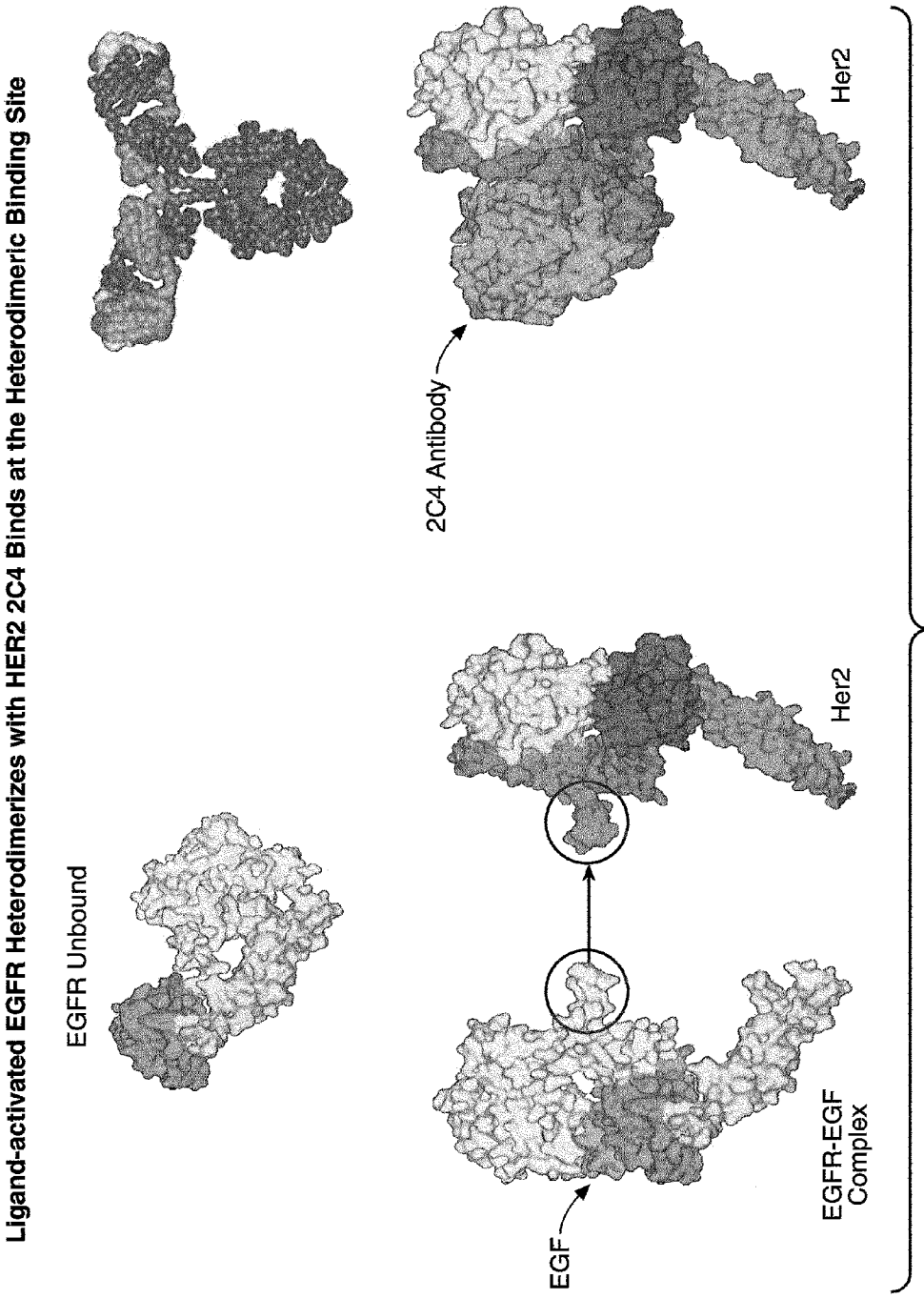

Ligand-activated EGFR Heterodimerizes with HER2 2C4 Binds at the Heterodimeric Binding Site
EGFR Unbound
2C4 Antibody
Her2
EGF
EGFR-EGF Complex
Her2

FIG. 4

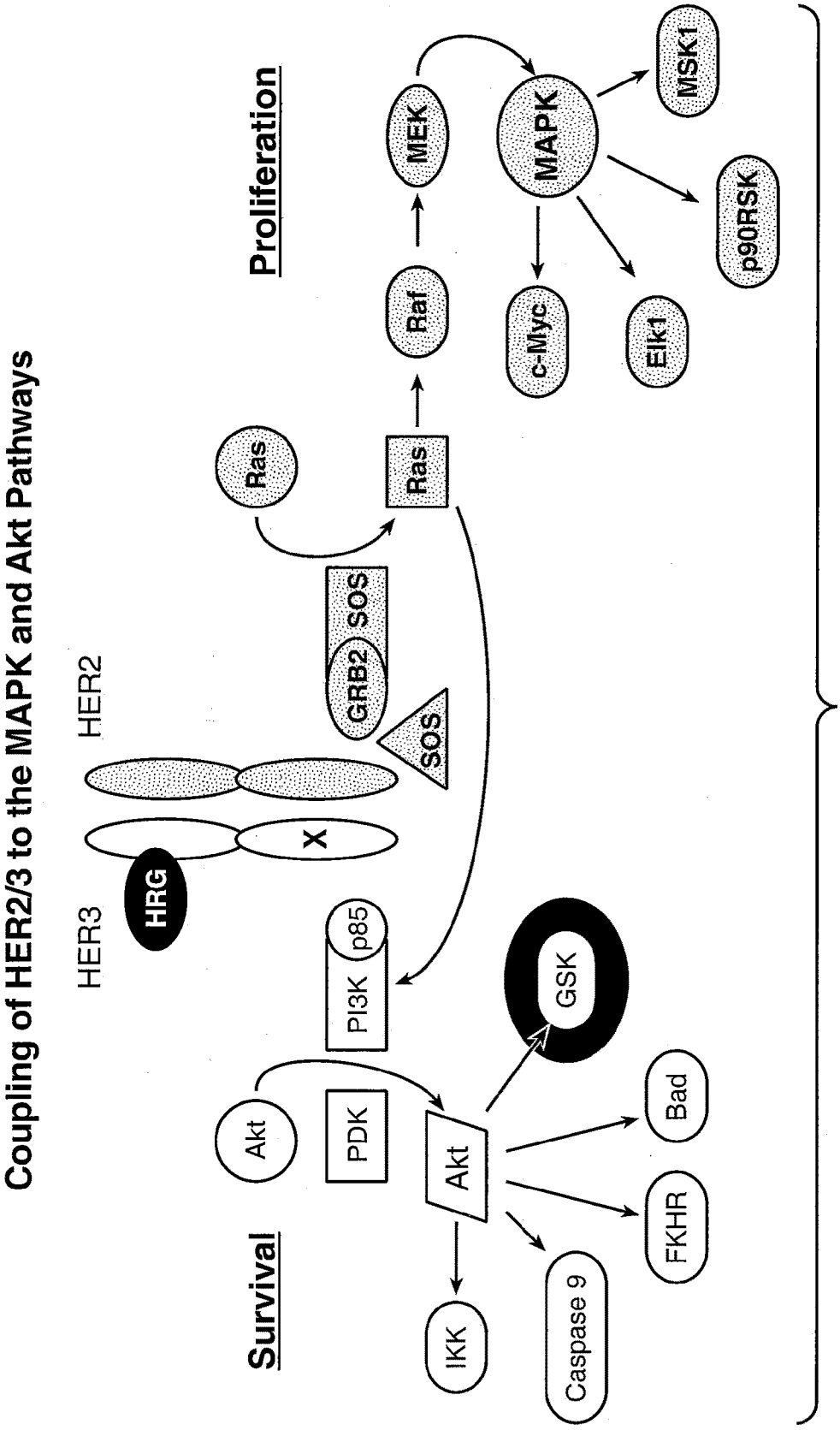
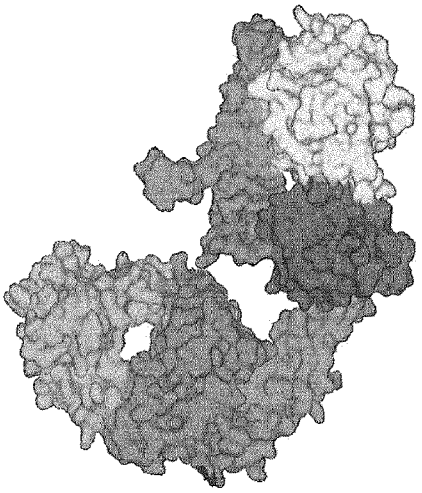

Coupling of HER2/3 to the MAPK and Akt Pathways
HER3
HER2
HRG
X
Ras
SOS
GRB2
SOS
Ras
Raf
MEK
MAPK
MSK1
p90RSK
c-Myc
Elk1
Proliferation
PI3K
p85
Akt
PDK
Akt
GSK
Bad
FKHR
IKK
Caspase 9
Survival

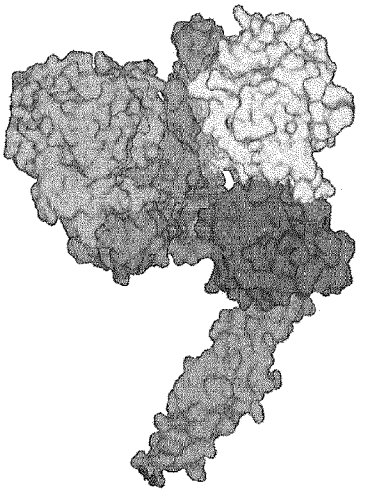
FIG. 5

Trastuzumab
Herceptin



- Binds in IV near JM.
- Protects against receptor shedding
- Moderately affects receptor down-modulation
- Slight effect on HER2's role as a coreceptor

Pertuzumab
Omnitarg



- Binds in II at dimerization interface
- Does not prevent receptor shedding
- Moderately affects receptor down-modulation
- Major effect on HER2's role as a coreceptor

FIG. 6

Light Chain

```
1   D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q D V N T A V A W Y Q Q K P G K A P K
46  L L I Y S A S F L Y S G V P S R F S G S R S G T D F T L T I S S L Q P E D F A T Y Y C Q Q
91  H Y T T P P T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L
136 L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T
181 L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
```

FIG. 7A

Heavy Chain

```
                15                  30                  45
1   EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGL
                60                  75                  90
46  EWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAED
               105                 120                 135
91  TAVYYCSRWGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSS
               150                 165                 180
136 KSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
               195                 210                 225
181 GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
               240                 255                 270
226 THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
               285                 300                 315
271 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD
               330                 345                 360
316 WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE
               375                 390                 405
361 MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
               420                 435                 449
406 SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
```

FIG. 7B

```
1                                                       15                                                      30                                                      45
V H S D I Q M T Q S P S S L S A S V G D R V T I T C K A S Q D V S I G V A W Y Q Q K P G K

46                                                      60                                                      75                                                      90
A P K L L I Y S A S Y R Y T G V P S R F S G S G S G T D F T L T I S S L Q P E D F A T Y Y

91                                                      105                                                     120                                                     135
C Q Q Y Y I Y P Y T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V

136                                                     150                                                     165                                                     180
V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S

181                                                     195                                                     210                    217
T L T L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
```

FIG. 8A

1 15 30 45
E V Q L V E S G G G L V Q P G G S L R L S C A A S G F T F T D Y T M D W V R Q A P G K G L

46 60 75 90
E W V A D V N P N S G G S I Y N Q R F K G R F T L S V D R S K N T L Y L Q M N S L R A E D

91 105 120 135
T A V Y Y C A R N L G P S F Y F D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S K

136 150 165 180
S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G

181 195 210 225
L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K T

226 240 255 270
H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H

271 285 300 315
E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W

316 330 345 360
L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E M

361 375 390 405
T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S

406 420 435 449
F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K

FIG. 8B

CHARACTERISTIC	420 mg	1050 mg	ALL
n	61	62	123
Median Age (Years)	59 (35-78)	56.5 (35-83)	57 (35-83)
ECOG PS 0 / 1 / 2-3	28 / 31 / 2	37 / 25 / 0	65 / 56 / 2
Measurable Disease	53 (87%)	59 (95%)	112 (91%)
Originally Platinum-Resistant	33 (54%)	33/61 (54%)	66 (54%)
Originally Platinum-Sensitive	28 (46%)	28/61 (46%)	56 (46%)
Median # Chemo Regimens	5.0 (1-10)	4.5 (0-13)	5.0 (0-13)
Median Duration of Ovarian Cancer (Months)	39.9 (3.3-146.7)	35.5 (10.5-443.7)	38.6 (3.3-443.7)

FIG. 9

ORGAN SYSTEM	420 mg (n=61)	1050 mg (n=62)	ALL (n=123)
- Any Adverse Events -	35 (57.4%)	33 (53.2%)	68 (55.3%)
Gastrointestinal	27 (44.3%)	16 (25.8%)	43 (35%)
- Diarrhea	-7 (11.5%)	-8 (12.9%)	-15 (12.2%)
- Intestinal Obstruction	-10 (16.4%)	-7 (11.3%)	-17 (13.8%)
Respiratory	8 (13.1%)	9 (14.5%)	17 (13.8%)
Fatigue, Asthenia	4 (6.6%)	2 (3.2%)	6 (4.9%)
Dehydration	4 (6.6%)	2 (3.2%)	6 (4.9%)
Cardiac	3 (4.9%)	2 (3.2%)	5 (4.1%)
Anorexia	2 (3.3%)	2 (3.2%)	4 (3.3%)
Infections	3 (4.9%)	0	3 (2.4%)

FIG. 10

PREFERRED TERM	420 mg (n=61)	1050 mg (n=62)	ALL (n=123)
- Any Adverse Events -	26 (42.6%)	18 (29.0%)	44 (35.8%)
Gastrointestinal	15 (24.6%)	8 (12.9%)	23 (18.7%)
- Diarrhea	-2 (3.3%)	-0 (0.0%)	-2 (1.6%)
- Intestinal Obstruction	-10 (16.4%)	-8 (12.9%)	-18 (14.6%)
Respiratory	4 (6.6%)	3 (4.8%)	7 (5.7%)
Cardiac	2 (3.3%)	2 (3.2%)	4 (3.3%)
Renal and Urinary	1 (1.6%)	3 (4.8%)	4 (3.3%)
Dehydration	1 (1.6%)	2 (3.2%)	3 (2.4%)
Infections	3 (4.9%)	0 (0.0%)	3 (2.4%)

FIG. 11

PREFERRED TERM	420 mg (n=61)	1050 mg (n=62)	ALL (n=123)
- Any Adverse Events -	4 (6.6%)	2 (3.2%)	6 (4.9%)
Diarrhea	1 (1.6%)	0	1 (0.8%)
Abdominal Pain	1 (1.6%)	0	1 (0.8%)
LVEF Decrease	0	1 (1.6%)	1 (0.8%)
Atrial Fibrillation	1 (1.6%)	0	1 (0.8%)
Pericardial Effusion	0	1 (1.6%)	1 (0.8%)
Pneumonia	1 (1.6%)	0	1 (0.8%)

FIG. 12

EVENT	420 mg (n=61)	1050 mg (n=62)	ALL (n=123)
Diarrhea *(All Grade 1-3)* - Grade 3	35 (57.4%) -7 (11.5%)	40 (64.5%) -8 (12.9%)	75 (61%) -15 (12.2%)
Rash & Skin Disorders *(All Grade 1-2)*	26 (42.6%)	31 (50%)	57 (46.3%)
LVEF Drop ≥ 10% Points *(None Confirmed by Central Read Thus Far)*	10/49 (20.4%)	13/50 (26.0%)	23/99 (23%)
LVEF Drop to < 50% *(None Confirmed by Central Read Thus Far)*	1/50 (2%)	4/50 (8%)	5/100 (5%)

FIG. 13

PREFERRED TERM	420 mg (n=61)	1050 mg (n=62)	ALL (n=123)
- Any Adverse Events -	13 (21.3%)	15 (24.2%)	28 (22.8%)
Ejection Fraction Decrease (>10% Points from Baseline)	10 (16.4%)	14 (22.6%)	24 (19.5%)
Ventricular Dysfunction	1 (1.6%)	0	1 (0.8%)
Atrial Fibrillation	1 (1.6%)	0	1 (0.8%)
Endocarditis Noninfective	1 (1.6%)	0	1 (0.8%)
Pericardial Effusion	0	1 (1.6%)	1 (0.8%)
Cardiac Tamponade	0	1 (1.6%)	1 (0.8%)

FIG. 14

ENDPOINT	420 mg	1050 mg	TOTAL
n	60	62	122
PR	2 (3.3%)	3 (4.8%)	5 (4.1%)
SD	23 (38.3%)	24 (38.7%)	47 (38.5%)
PD	27 (45.0%)	29 (46.8%)	56 (45.9%)
Response UTD	2 (3.3%)	2 (3.2%)	4 (3.3%)
Median TTP (Weeks)	7.0	6.6	6.6
Median Survival (Weeks)	40.1	–	40.1

FIG. 15

CA-125 RESPONSE	420 mg	1050 mg	ALL
>50% Reduction	7 (1 PR, **4 SD**, 2 PD)	5 (1 PR, **3 SD**, 1 PD)	12 (2 PR, **7 SD**, 3 PD)
>25% but <50% Reduction in CA-125	3 (**3 SD**)	5 (**3 SD**, 1 PD, 1 UE)	8 (**6 SD**, 1 PD, 1 UE)
<25% Reduction	6 (1 PR, **4 SD**, 1 PD)	10 (1 PR, **8 SD**, 1 PD)	16 (2 PR, **12 SD**, 2 PD)
No Response	37 (12 SD, 20 PD, 1 UE, 4 None)	36 (1 PR, **10 SD**, 24 PD, 1 None)	73 (1 PR, **22 SD**, 44 PD, 1 UE, 5 None)
Total	53	56	109

FIG. 18

Patients Treated	61
Evaluable for Efficacy	54
	ELISA (>30% Tumor)
pHER2 Data (n=65 Biopsies)	34
pHER2+	10
% pHER2+	29%
Evaluable pts *and* pHER2 Data	31
pHER2+	8
% pHER2+	26%

FIG. 19

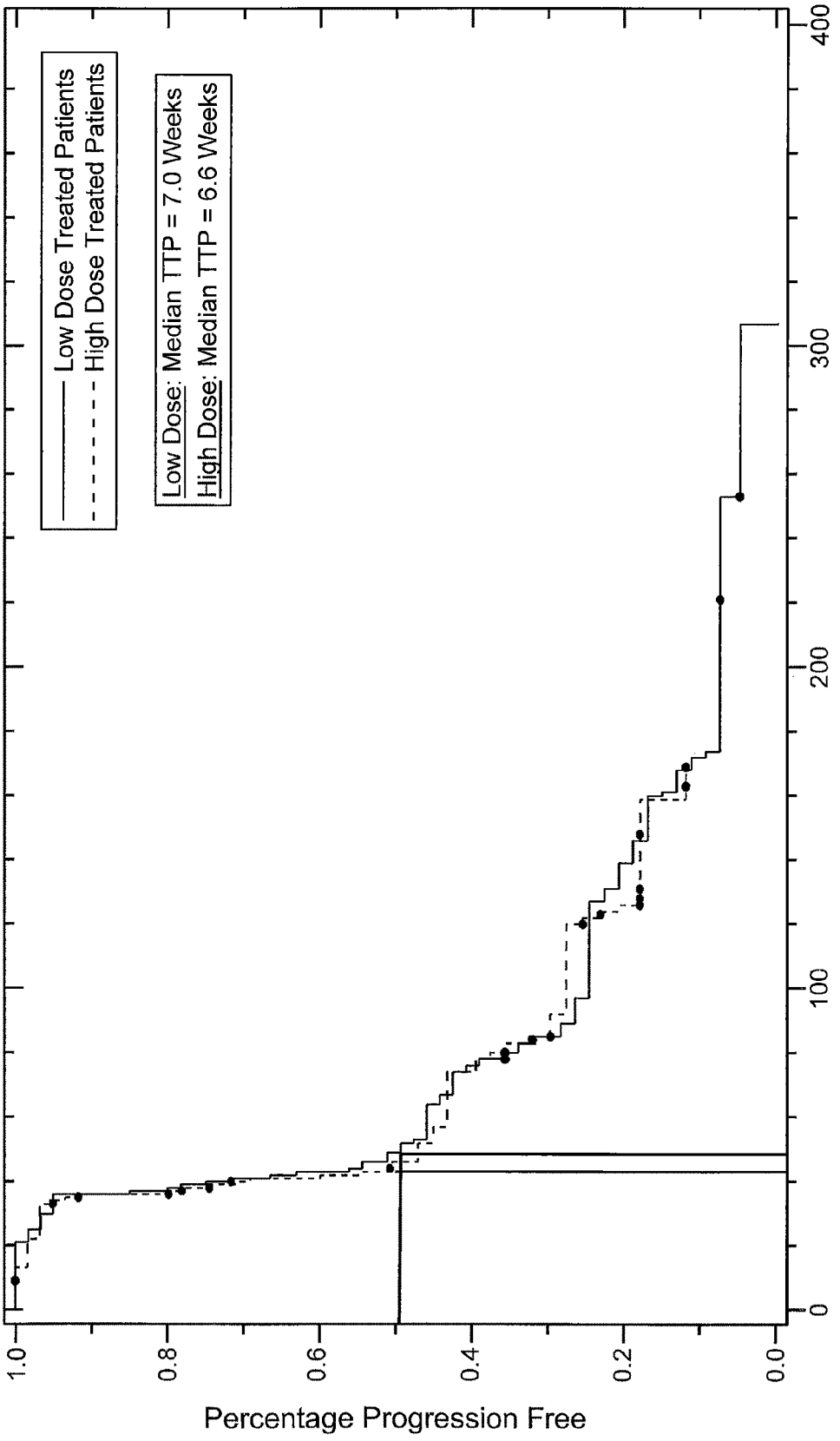

Low Dose Treated Patients
High Dose Treated Patients
Low Dose: Median TTP = 7.0 Weeks
High Dose: Median TTP = 6.6 Weeks
Percentage Progression Free
1.0
0.8
0.6
0.4
0.2
0.0
Time to Disease Progression (Days)
0
100
200
300
400

FIG. 16

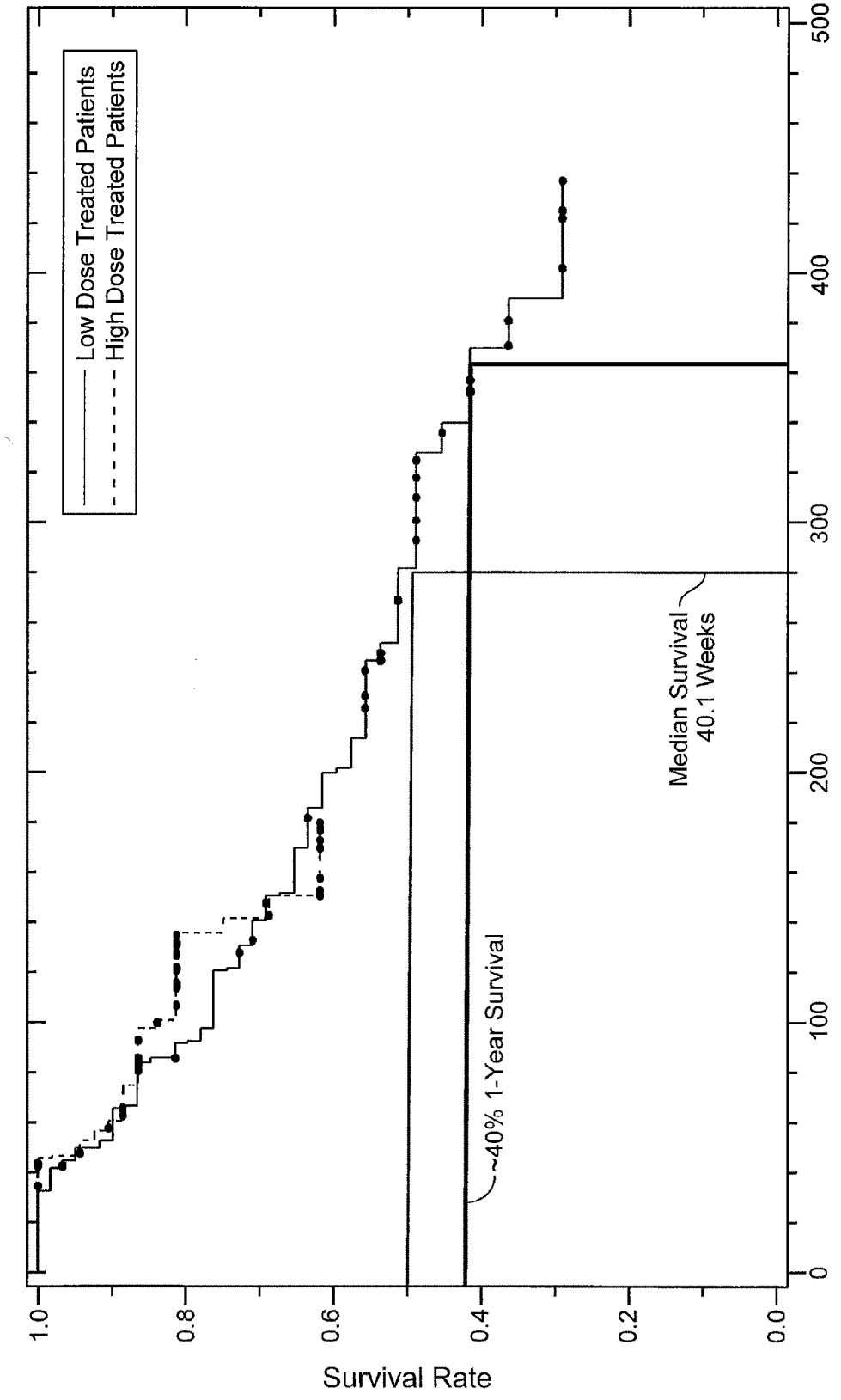

Low Dose Treated Patients
High Dose Treated Patients
Median Survival
40.1 Weeks
~40% 1-Year Survival
Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0
Survival (Days)
0
100
200
300
400
500

FIG. 17

	420 mg pHER2+	420 mg pHER2-	420 mg pHER2 ?
N	8	23	29
PR	1 (12.5%)	0 (0.0%)	1 (3.4%)
SD	5 (62.5%)	5 (21.7%)	13 (44.8%)
PD	2 (25.0%)	13 (56.5%)	12 (41.4%)
Response UTD	0 (0.0%)	2 (8.7%)	0 (0.0%)
Median TTP (Weeks)	20.9	6.0	9.1
Median Survival (Weeks)	–	35.9	48.4

FIG. 20

Best Response	Reduction in BSLD	Reduction in CA-125	Weeks on Treatment	pHER2 ELISA
PR	68%	54%	25	+
PR	78%	22%	23	NA
SD		33%	36	NA
SD		57%	20	NA
SD		15%	19	-
SD		60%	32	-
SD		None	21	+
SD		21%	44+	NA
SD		None	23	NA
SD		62%	24	+
SD		5%	36	-
SD		56%	18	NA
SD		None	25	NA
Mixed	30%	None	13	NA

FIG. 21

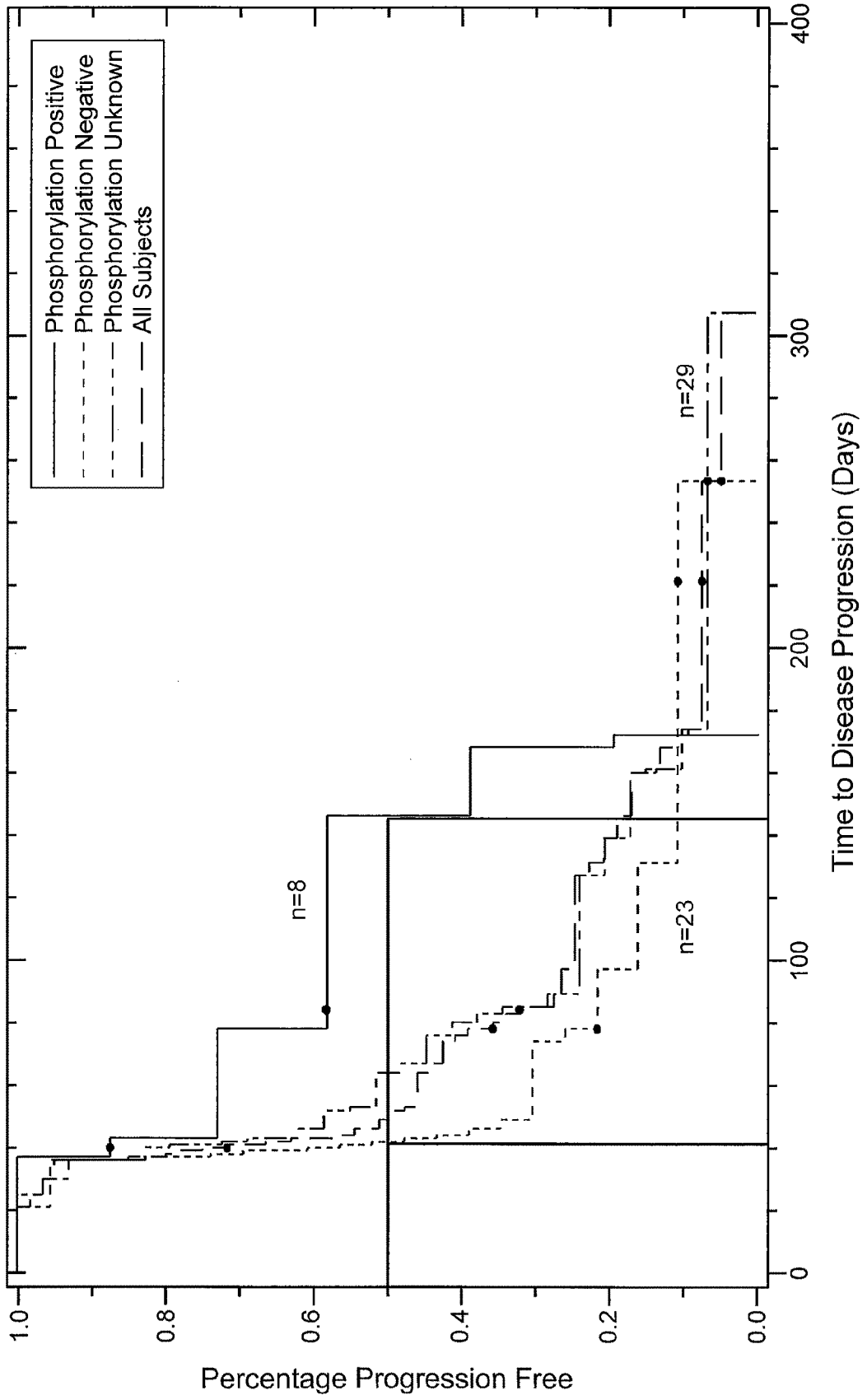

Phosphorylation Positive
Phosphorylation Negative
Phosphorylation Unknown
All Subjects
n=8
n=23
n=29
Time to Disease Progression (Days)
0
100
200
300
400
Percentage Progression Free
1.0
0.8
0.6
0.4
0.2
0.0

FIG. 22

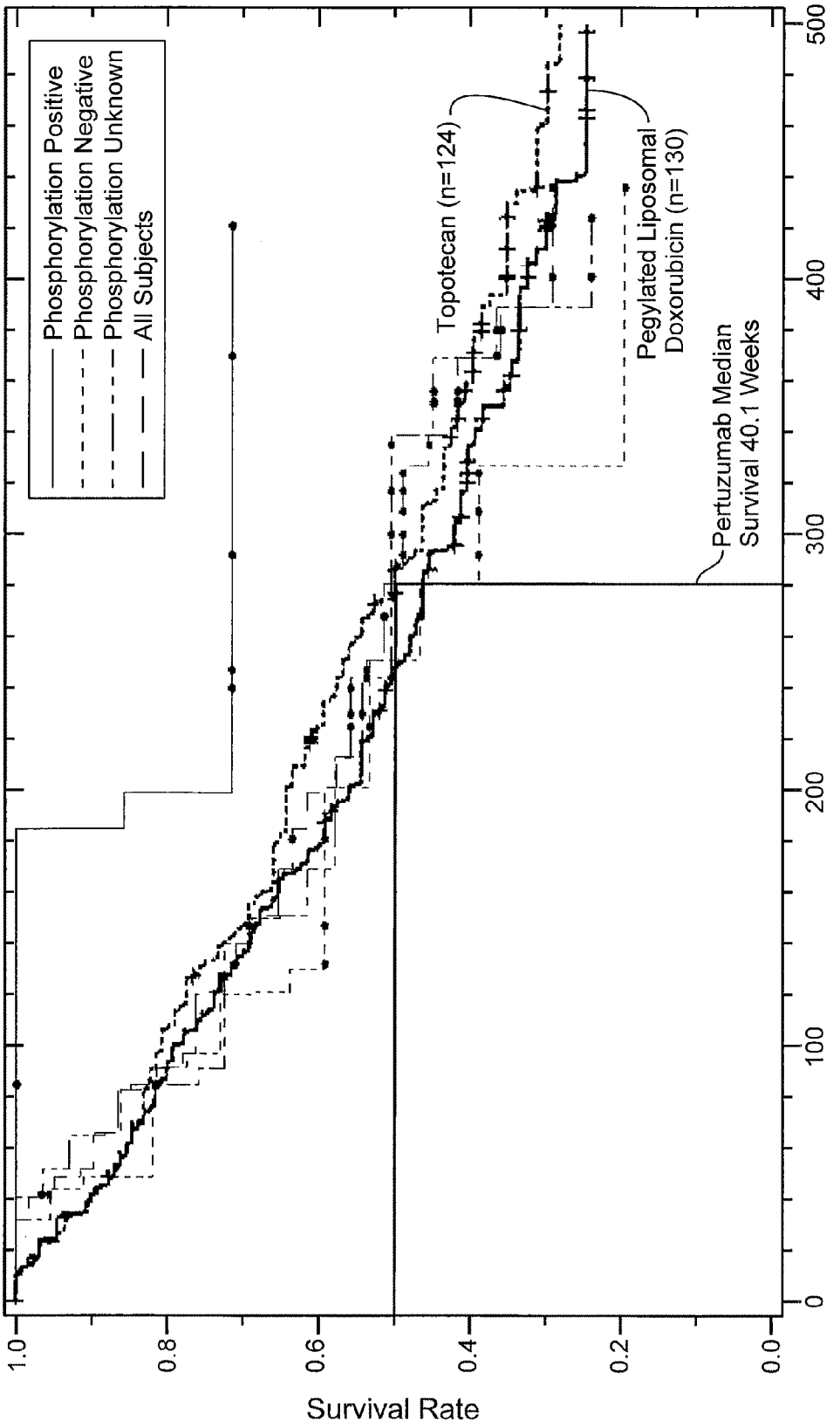

Phosphorylation Positive
Phosphorylation Negative
Phosphorylation Unknown
All Subjects
Topotecan (n=124)
Pegylated Liposomal Doxorubicin (n=130)
Pertuzumab Median Survival 40.1 Weeks
Survival (Days)
0
100
200
300
400
500
Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0

FIG. 23

EXTENDING TIME TO DISEASE PROGRESSION OR SURVIVAL IN CANCER PATIENTS

This is a continuation application which claims priority to continuation application Ser. No. 12/271,564, filed Nov. 14, 2008 (now abandoned), which claims priority under 35 USC §120 to non-provisional application Ser. No. 11/359,185, filed Feb. 21, 2006 (now abandoned), which claims priority under 35 USC §119 to provisional application No. 60/655,277, filed Feb. 23, 2005, the entire disclosures of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns extending time to disease progression or survival in a cancer patient, where the patient's cancer displays HER activation, by treating the patient with a HER dimerization inhibitor, such as pertuzumab.

BACKGROUND OF THE INVENTION

HER Receptors and Antibodies Thereagainst

The HER family of receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes four distinct members including epidermal growth factor receptor (EGFR, ErbB1, or HER1), HER2 (ErbB2 or p185^{neu}), HER3 (ErbB3) and HER4 (ErbB4 or tyro2).

EGFR, encoded by the erbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, transforming growth factor alpha (TGF-α), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn *Pharmac. Ther.* 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-α and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn, supra; Masui et al. *Cancer Research* 44:1002-1007 (1984); and Wu et al. *J. Clin. Invest.* 95:1897-1905 (1995).

The second member of the HER family, p185^{neu}, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., *Science,* 235:177-182 (1987); Slamon et al., *Science,* 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. See, among others, King et al., *Science,* 229:974 (1985); Yokota et al., *Lancet:* 1:765-767 (1986); Fukushige et al., *Mol Cell Biol.,* 6:955-958 (1986); Guerin et al., *Oncogene Res.,* 3:21-31 (1988); Cohen et al., *Oncogene,* 4:81-88 (1989); Yonemura et al., *Cancer Res.,* 51:1034 (1991); Borst et al., *Gynecol. Oncol.,* 38:364 (1990); Weiner et al., *Cancer Res.,* 50:421-425 (1990); Kern et al., *Cancer Res.,* 50:5184 (1990); Park et al., *Cancer Res.,* 49:6605 (1989); Zhau et al., *Mol. Carcinog.,* 3:254-257 (1990); Aasland et al. *Br. J. Cancer* 57:358-363 (1988); Williams et al. *Pathobiology* 59:46-52 (1991); and McCann et al., *Cancer,* 65:88-92 (1990). HER2 may be overexpressed in prostate cancer (Gu et al. *Cancer Lett.* 99:185-9 (1996); Ross et al. *Hum. Pathol.* 28:827-33 (1997); Ross et al. *Cancer* 79:2162-70 (1997); and Sadasivan et al. *J. Urol.* 150:126-31 (1993)).

Antibodies directed against the rat p185^{neu} and human HER2 protein products have been described.

Drebin and colleagues have raised antibodies against the rat neu gene product, p185^{neu} See, for example, Drebin et al., *Cell* 41:695-706 (1985); Myers et al., *Meth. Enzym.* 198:277-290 (1991); and WO94/22478. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions of p185^{neu} result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. See also U.S. Pat. No. 5,824,311 issued Oct. 20, 1998.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of HER2 antibodies which were characterized using the human breast tumor cell line SK-BR-3. Relative cell proliferation of the SK-BR-3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also U.S. Pat. No. 5,677,171 issued Oct. 14, 1997. The HER2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis et al. *Cancer Research* 56:1457-1465 (1996); and Schaefer et al. *Oncogene* 15:1385-1394 (1997).

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein.

Other HER2 antibodies with various properties have been described in Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991); McKenzie et al. *Oncogene* 4:543-548 (1989); Maier et al. *Cancer Res.* 51:5361-5369 (1991); Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al. *PNAS (USA)* 88:8691-8695 (1991); Bacus et al. *Cancer Research* 52:2580-2589 (1992); Xu et al. *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992); Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994);

Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al. *Oncogene* 14:2099-2109 (1997).

Homology screening has resulted in the identification of two other HER receptor family members; HER3 (U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS (USA)* 86:9193-9197 (1989)) and HER4 (EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The HER receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of HER ligands (Earp et al. *Breast Cancer Research and Treatment* 35: 115-132 (1995)). EGFR is bound by six different ligands; epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin, heparin binding epidermal growth factor (HB-EGF), betacellulin and epiregulin (Groenen et al. *Growth Factors* 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta and gamma heregulins (Holmes et al., *Science,* 256:1205-1210 (1992); U.S. Pat. No. 5,641,869; and Schaefer et al. *Oncogene* 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motor neuron derived factor (SMDF). For a review, see Groenen et al. *Growth Factors* 11:235-257 (1994); Lemke, G. *Molec. & Cell. Neurosci.* 7:247-262 (1996) and Lee et al. *Pharm. Rev.* 47:51-85 (1995). Recently three additional HER ligands were identified; neuregulin-2 (NRG-2) which is reported to bind either HER3 or HER4 (Chang et al. *Nature* 387 509-512 (1997); and Carraway et al *Nature* 387:512-516 (1997)); neuregulin-3 which binds HER4 (Zhang et al. *PNAS* (USA) 94(18):9562-7 (1997)); and neuregulin-4 which binds HER4 (Harari et al. *Oncogene* 18:2681-89 (1999)) HB-EGF, betacellulin and epiregulin also bind to HER4.

While EGF and TGFα do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase. See Earp et al., *supra. Likewise, when HER*3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski et al., *J. Biol. Chem.,* 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi et al., *Journal of Neuroscience* 15: 1329-1340 (1995); Morrissey et al., *Proc. Natl. Acad. Sci. USA* 92: 1431-1435 (1995); and Lewis et al., *Cancer Res.,* 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, *Cell* 78:5-8 (1994)).

Patent publications related to HER antibodies include: U.S. Pat. No. 5,677,171, U.S. Pat. No. 5,720,937, U.S. Pat. No. 5,720,954, U.S. Pat. No. 5,725,856, U.S. Pat. No. 5,770,195, U.S. Pat. No. 5,772,997, U.S. Pat. No. 6,165,464, U.S. Pat. No. 6,387,371, U.S. Pat. No. 6,399,063, US2002/0192211A1, U.S. Pat. No. 6,015,567, U.S. Pat. No. 6,333,169, U.S. Pat. No. 4,968,603, U.S. Pat. No. 5,821,337, U.S. Pat. No. 6,054,297, U.S. Pat. No. 6,407,213, U.S. Pat. No. 6,719,971, U.S. Pat. No. 6,800,738, US2004/0236078A1, U.S. Pat. No. 5,648,237, U.S. Pat. No. 6,267,958, U.S. Pat. No. 6,685,940, U.S. Pat. No. 6,821,515, WO98/17797, U.S. Pat. No. 6,127,526, U.S. Pat. No. 6,333,398, U.S. Pat. No. 6,797,814, U.S. Pat. No. 6,339,142, U.S. Pat. No. 6,417,335, U.S. Pat. No. 6,489,447, WO99/31140, US2003/0147884A1, US2003/0170234A1, US2005/0002928A1, U.S. Pat. No. 6,573,043, US2003/0152987A1, WO99/48527, US2002/0141993A1, WO01/00245, US2003/0086924, US2004/0013667A1, WO00/69460, WO01/00238, WO01/15730, U.S. Pat. No. 6,627,196B1, U.S. Pat. No. 6,632,979B1, WO01/00244, US2002/0090662A1, WO01/89566, US2002/0064785, US2003/0134344, WO 04/24866, US2004/0082047, US2003/0175845A1, WO03/087131, US2003/0228663, WO2004/008099A2, US2004/0106161, WO2004/048525, US2004/0258685A1, U.S. Pat. No. 5,985,553, U.S. Pat. No. 5,747,261, U.S. Pat. No. 4,935,341, U.S. Pat. No. 5,401,638, U.S. Pat. No. 5,604,107, WO 87/07646, WO 89/10412, WO 91/05264, EP 412,116 B1, EP 494,135 B1, U.S. Pat. No. 5,824,311, EP 444,181 B1, EP 1,006,194 A2, US 2002/0155527A1, WO 91/02062, U.S. Pat. No. 5,571,894, U.S. Pat. No. 5,939,531, EP 502,812 B1, WO 93/03741, EP 554,441 B1, EP 656,367 A1, U.S. Pat. No. 5,288,477, U.S. Pat. No. 5,514,554, U.S. Pat. No. 5,587,458, WO 93/12220, WO 93/16185, U.S. Pat. No. 5,877,305, WO 93/21319, WO 93/21232, U.S. Pat. No. 5,856,089, WO 94/22478, U.S. Pat. No. 5,910,486, U.S. Pat. No. 6,028,059, WO 96/07321, U.S. Pat. No. 5,804,396, U.S. Pat. No. 5,846,749, EP 711,565, WO 96/16673, U.S. Pat. No. 5,783,404, U.S. Pat. No. 5,977,322, U.S. Pat. No. 6,512,097, WO 97/00271, U.S. Pat. No. 6,270,765, U.S. Pat. No. 6,395,272, U.S. Pat. No. 5,837,243, WO 96/40789, U.S. Pat. No. 5,783,186, U.S. Pat. No. 6,458,356, WO 97/20858, WO 97/38731, U.S. Pat. No. 6,214,388, U.S. Pat. No. 5,925,519, WO 98/02463, U.S. Pat. No. 5,922,845, WO 98/18489, WO 98/33914, U.S. Pat. No. 5,994,071, WO 98/45479, U.S. Pat. No. 6,358,682 B1, US 2003/0059790, WO 99/55367, WO 01/20033, US 2002/0076695 A1, WO 00/78347, WO 01/09187, WO 01/21192, WO 01/32155, WO 01/53354, WO 01/56604, WO 01/76630, WO02/05791, WO 02/11677, U.S. Pat. No. 6,582,919, US2002/0192652A1, US 2003/0211530A1, WO 02/44413, US 2002/0142328, U.S. Pat. No. 6,602,670 B2, WO 02/45653, WO 02/055106, US 2003/0152572, US 2003/0165840, WO 02/087619, WO 03/006509, WO03/012072, WO 03/028638, US 2003/0068318, WO 03/041736, EP 1,357,132, US 2003/0202973, US 2004/0138160, U.S. Pat. No. 5,705,157, U.S. Pat. No. 6,123,939, EP 616,812 B1, US 2003/0103973, US 2003/0108545, U.S. Pat. No. 6,403,630 B1, WO 00/61145, WO 00/61185, U.S. Pat. No. 6,333,348 B1, WO 01/05425, WO 01/64246, US 2003/0022918, US 2002/0051785 A1, U.S. Pat. No. 6,767,541, WO 01/76586, US 2003/0144252, WO 01/87336, US 2002/0031515 A1, WO 01/87334, WO 02/05791, WO 02/09754, US 2003/0157097, US 2002/0076408, WO 02/055106, WO 02/070008, WO 02/089842 and WO 03/86467.

Diagnostics

Patients treated with the HER2 antibody trastuzumab are selected for therapy based on HER2 overexpression/amplification. See, for example, WO99/31140 (Paton et al.), US2003/0170234A1 (Hellmann, S.), and US2003/0147884 (Paton et al.); as well as WO01/89566, US2002/0064785, and US2003/0134344 (Mass et al.). See, also, US2003/0152987, Cohen et al., concerning immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) for detecting HER2 overexpression and amplification.

WO2004/053497 and US2004/024815A1 (Bacus et al.), as well as US 2003/0190689 (Crosby and Smith), refer to determining or predicting response to trastuzumab therapy.

US2004/013297A1 (Bacus et al.) concerns determining or predicting response to ABX0303 EGFR antibody therapy. WO2004/000094 (Bacus et al.) is directed to determining response to GW572016, a small molecule, EGFR-HER2 tyrosine kinase inhibitor. WO2004/063709, Amler et al., refers to biomarkers and methods for determining sensitivity to EGFR inhibitor, erlotinib HCl. US2004/0209290, Cobleigh et al., concerns gene expression markers for breast cancer prognosis. Patients treated with pertuzumab can be selected for therapy based on HER activation or dimerization. Patent publications concerning pertuzumab and selection of patients for therapy therewith include: WO01/00245 (Adams et al.); US2003/0086924 (Sliwkowski, M.); US2004/0013667A1 (Sliwkowski, M.); as well as WO2004/008099A2, and US2004/0106161 (Bossenmaier et al.).

Cronin et al. *Am. J. Path.* 164(1): 35-42 (2004) describes measurement of gene expression in archival paraffin-embedded tissues. Ma et al. *Cancer Cell* 5:607-616 (2004) describes gene profiling by gene oligonucleotide microarray using isolated RNA from tumor-tissue sections taken from archived primary biopsies.

Pertuzumab (also known as recombinant human monoclonal antibody 2C4; OMNITARG™, Genentech, Inc, South San Francisco) represents the first in a new class of agents known as HER dimerization inhibitors (HDI) and functions to inhibit the ability of HER2 to form active heterodimers with other HER receptors (such as EGFR/HER1, HER3 and HER4) and is active irrespective of HER2 expression levels. See, for example, Harari and Yarden *Oncogene* 19:6102-14 (2000); Yarden and Sliwkowski. *Nat. Rev Mol Cell Biol* 2:127-37 (2001); Sliwkowski *Nat Struct Biol* 10:158-9 (2003); Cho et al. *Nature* 421:756-60 (2003); and Malik et al. *Pro Am Soc Cancer Res* 44:176-7 (2003).

Pertuzumab blockade of the formation of HER2-HER3 heterodimers in tumor cells has been demonstrated to inhibit critical cell signaling, which results in reduced tumor proliferation and survival (Agus et al. *Cancer Cell* 2:127-37 (2002)).

Pertuzumab has undergone testing as a single agent in the clinic with a phase Ia trial in patients with advanced cancers and phase II trials in patients with ovarian cancer and breast cancer as well as lung and prostate cancer. In a Phase I study, patients with incurable, locally advanced, recurrent or metastatic solid tumors that had progressed during or after standard therapy were treated with pertuzumab given intravenously every 3 weeks. Pertuzumab was generally well tolerated. Tumor regression was achieved in 3 of 20 patients evaluable for response. Two patients had confirmed partial responses. Stable disease lasting for more than 2.5 months was observed in 6 of 21 patients (Agus et al. *Pro Am Soc Clin Oncol* 22:192 (2003)). At doses of 2.0-15 mg/kg, the pharmacokinetics of pertuzumab was linear, and mean clearance ranged from 2.69 to 3.74 mL/day/kg and the mean terminal elimination half-life ranged from 15.3 to 27.6 days. Antibodies to pertuzumab were not detected (Allison et al. *Pro Am Soc Clin Oncol* 22:197 (2003)).

SUMMARY OF THE INVENTION

The present invention provides the clinical data from human cancer patients treated with a HER dimerization inhibitor, pertuzumab. Patients were evaluated for HER activation, as determined using a phospho-ELISA bioassay. Clinical benefit, as measured by time to disease progression (TTP) and survival, was observed in patients displaying HER activation.

Accordingly, the invention provides a method for extending time to disease progression (TTP) or survival in a cancer patient comprising administering a HER dimerization inhibitor to the patient in an amount which extends TTP or survival in the patent, wherein the patient's cancer displays HER activation.

The invention also concerns a method for extending time to disease progression (TTP) or survival in a patient with ovarian, peritoneal, or fallopian tube cancer comprising administering pertuzumab to the patient in an amount which extends TTP or survival in the patent, wherein the patient's cancer displays HER2 activation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** provides a schematic of the HER2 protein structure, and amino acid sequences for Domains I-IV (SEQ ID Nos. 19-22, respectively) of the extracellular domain thereof.

FIGS. **2**A and **2**B depict alignments of the amino acid sequences of the variable light (V_L) (FIG. **2**A) and variable heavy (V_H) (FIG. **2**B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 1 and 2, respectively); V_L and V_H domains of variant 574/pertuzumab (SEQ ID Nos. 3 and 4, respectively), and human V_L and V_H consensus frameworks (hum xi, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 5 and 6, respectively). Asterisks identify differences between variable domains of pertuzumab and murine monoclonal antibody 2C4 or between variable domains of pertuzumab and the human framework. Complementarity Determining Regions (CDRs) are in brackets.

FIGS. **3**A and **3**B show the amino acid sequences of pertuzumab light chain (FIG. **3**A; SEQ ID NO. 13) and heavy chain (FIG. **3**B; SEQ ID No. 14). CDRs are shown in bold. Calculated molecular mass of the light chain and heavy chain are 23,526.22 Da and 49,216.56 Da (cysteines in reduced form). The carbohydrate moiety is attached to Asn 299 of the heavy chain.

FIG. **4** depicts, schematically, binding of 2C4 at the heterodimeric binding site of HER2, thereby preventing heterodimerization with activated EGFR or HER3.

FIG. **5** depicts coupling of HER2/HER3 to the MAPK and Akt pathways.

FIG. **6** compares various activities of trastuzumab and pertuzumab.

FIGS. **7**A and **7**B show the amino acid sequences of trastuzumab light chain (FIG. **7**A; SEQ ID No. 15) and heavy chain (FIG. **7**B; SEQ ID No. 16), respectively.

FIGS. **8**A and **8**B depict a variant pertuzumab light chain sequence (FIG. **8**A; SEQ ID No. 17) and a variant pertuzumab heavy chain sequence (FIG. **8**B; SEQ ID No. 18), respectively.

FIG. **9** provides baseline demographics of patients treated in Example 1.

FIG. **10** shows all grade 3-4 adverse events (irrespective of relatedness to treatment).

FIG. **11** shows serious adverse events (irrespective or relatedness to treatment).

FIG. **12** summarizes serious adverse events judged to be related to study drug by investigators.

FIG. **13** provides information on selected adverse events.

FIG. **14** depicts cardiac serious adverse events and adverse events requiring expedited reporting.

FIG. **15** summarizes efficacy results for the phase II study of pertuzumab in Example 1.

FIG. **16** shows time to disease progression (TTP) efficacy for evaluable ovarian cancer subjects treated with either a low dose (420 mg) or high dose (1050 mg) of pertuzumab.

FIG. **17** shows overall survival efficacy for evaluable ovarian cancer subjects treated with either low dose (420 mg) or high dose (1050 mg) of pertuzumab. Historical median survival for ovarian cancer subjects treated with topotecan was 43 weeks, and for liposomal doxorubicin was 36 weeks.

FIG. **18** provides CA-125 responses for ovarian cancer subjects treated with either 420 mg or 1050 mg of pertuzumab.

FIG. **19** provides phospho-HER2 (pHER2) status, as determined by ELISA, for ovarian cancer subjects treated with 420 mg of pertuzumab.

FIG. **20** provides clinical efficacy results by pHER2 status, as determined by ELISA, for ovarian cancer subjects treated with 420 mg of pertuzumab.

FIG. **21** provides pHER2 status, as determined by ELISA, for ovarian patients treated with 420 mg of pertuzumab showing evidence of activity (partial response, PR, or stable disease, SD, for greater than 18 weeks). BSLD refers to baseline sum of longest diameter.

FIG. **22** shows TTP efficacy by pHER2 status. Ovarian cancer subjects were treated with 420 mg of pertuzumab. Overall TTP was 6.6 weeks; TTP in pHER positive subjects was 20.9 weeks; TTP in pHER2 negative subjects was 6.0 weeks; and TTP in subjects with unknown pHER2 status was 9.1 weeks.

FIG. **23** depicts overall survival by pHER2 status. Ovarian cancer subjects were treated with 420 mg of pertuzumab. Historical median survival for ovarian cancer subjects treated with topotecan was 43 weeks, and for liposomal doxorubicin was 36 weeks.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

Herein "time to disease progression" or "TTP" refer to the time, generally measured in weeks or months, from the time of initial treatment (e.g. with a HER dimerization inhibitor, such as pertuzumab), until the cancer progresses or worsens. Such progression can be evaluated by the skilled clinician. In the case of ovarian cancer, for instance, progression can be evaluated by RECIST (see, for example, Therasse et al., *J. Nat. Cancer Inst.* 92(3): 205-216 (2000)).

By "extending TTP" is meant increasing the time to disease progression in a treated patient relative to an untreated patient (i.e. relative to a patient not treated with a HER dimerization inhibitor, such as pertuzumab), or relative to a patient who does not display HER activation, and/or relative to a patient treated with an approved anti-tumor agent (such as topotecan or liposomal doxorubicin, where the cancer is ovarian cancer).

"Survival" refers to the patient remaining alive, and includes overall survival as well as progression free survival.

"Overall survival" refers to the patient remaining alive for a defined period of time, such as 1 year, 5 years, etc from the time of diagnosis or treatment.

"Progression free survival" refers to the patient remaining alive, without the cancer progressing or getting worse.

By "extending survival" is meant increasing overall or progression free survival in a treated patient relative to an untreated patient (i.e. relative to a patient not treated with a HER dimerization inhibitor, such as pertuzumab), or relative to a patient who does not display HER activation, and/or relative to a patient treated with an approved anti-tumor agent (such as topotecan or liposomal doxorubicin, where the cancer is ovarian cancer).

An "objective response" refers to a measurable response, including complete response (CR) or partial response (PR).

By "complete response" or "CR" is intended the disappearance of all signs of cancer in response to treatment. This does not always mean the cancer has been cured.

"Partial response" or "PR" refers to a decrease in the size of one or more tumors or lesions, or in the extent of cancer in the body, in response to treatment.

A "HER receptor" is a receptor protein tyrosine kinase which belongs to the HER receptor family and includes EGFR, HER2, HER3 and HER4 receptors. The HER receptor will generally comprise an extracellular domain, which may bind an HER ligand and/or dimerize with another HER receptor molecule; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The HER receptor may be a "native sequence" HER receptor or an "amino acid sequence variant" thereof. Preferably the HER receptor is native sequence human HER receptor.

The terms "ErbB1," "HER1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881-914 (1987), including naturally occurring mutant forms thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS (USA)* 87:4207-4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product.

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to human HER2 protein described, for example, in Semba et al., *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). The term "erbB2" refers to the gene encoding human ErbB2 and "neu" refers to the gene encoding rat $p185^{neu}$. Preferred HER2 is native sequence human HER2.

Herein, "HER2 extracellular domain" or "HER2 ECD" refers to a domain of HER2 that is outside of a cell, either anchored to a cell membrane, or in circulation, including fragments thereof. In one embodiment, the extracellular domain of HER2 may comprise four domains: "Domain I" (amino acid residues from about 1-195; SEQ ID NO:19), "Domain H" (amino acid residues from about 196-319; SEQ ID NO:20), "Domain III" (amino acid residues from about 320-488: SEQ ID NO:21), and "Domain IV" (amino acid residues from about 489-630; SEQ ID NO:22) (residue numbering without signal peptide). See Garrett et al. *Mol. Cell.* 11: 495-505 (2003), Cho et al. *Nature* 421: 756-760 (2003), Franklin et al. *Cancer Cell* 5:317-328 (2004), and Plowman et al. *Proc. Natl. Acad. Sci.* 90:1746-1750 (1993), as well as FIG. **1** herein.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS (USA)* 86:9193-9197 (1989).

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-475 (1993), including isoforms thereof, e.g., as disclosed in WO99/19488, published Apr. 22, 1999.

By "HER ligand" is meant a polypeptide which binds to and/or activates a HER receptor. The HER ligand of particular interest herein is a native sequence human HER ligand such as epidermal growth factor (EGF) (Savage et al., *J. Biol. Chem.* 247:7612-7621 (1972)); transforming growth factor alpha (TGF-α) (Marquardt et al., *Science* 223:1079-1082 (1984)); amphiregulin also known as schwanoma or keratinocyte autocrine growth factor (Shoyab et al. *Science* 243:1074-

1076 (1989); Kimura et al. *Nature* 348:257-260 (1990); and Cook et al. *Mol. Cell. Biol.* 11:2547-2557 (1991)); betacellulin (Shing et al., *Science* 259:1604-1607 (1993); and Sasada et al. *Biochem. Biophys. Res. Commun.* 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., *Science* 251:936-939 (1991)); epiregulin (Toyoda et al., *J. Biol. Chem.* 270:7495-7500 (1995); and Komurasaki et al. *Oncogene* 15:2841-2848 (1997)); a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., *Nature* 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., *Proc. Natl. Acad. Sci.* 94:9562-9567 (1997)); neuregulin-4 (NRG-4) (Harari et al. *Oncogene* 18:2681-89 (1999)); and cripto (CR-1) (Kannan et al. *J. Biol. Chem.* 272(6):3330-3335 (1997)). HER ligands which bind EGFR include EGF, TGF-α, amphiregulin, betacellulin, HB-EGF and epiregulin. HER ligands which bind HER3 include heregulins. HER ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3, NRG-4, and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide encoded by the heregulin gene product as disclosed in U.S. Pat. No. 5,641,869, or Marchionni et al., *Nature,* 362:312-318 (1993). Examples of heregulins include heregulin-α, heregulin-β1, heregulin-β2 and heregulin-β3 (Holmes et al., *Science,* 256:1205-1210 (1992); and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. *Cell* 69:205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. *Cell* 72:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al., *Nature,* 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho et al. *J. Biol. Chem.* 270:14523-14532 (1995)); γ-heregulin (Schaefer et al. *Oncogene* 15:1385-1394 (1997)).

A "HER dimer" herein is a noncovalently associated dimer comprising at least two HER receptors. Such complexes may form when a cell expressing two or more HER receptors is exposed to an HER ligand and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20):14661-14665 (1994), for example. Other proteins, such as a cytokine receptor subunit (e.g. gp130) may be associated with the dimer. Preferably, the HER dimer comprises HER2.

A "HER heterodimer" herein is a noncovalently associated heterodimer comprising at least two different HER receptors, such as EGFR-HER2, HER2-HER3 or HER2-HER4 heterodimers.

A "HER inhibitor" is an agent which interferes with HER activation or function. Examples of HER inhibitors include HER antibodies (e.g. EGFR, HER2, HER3, or HER4 antibodies); EGFR-targeted drugs; small molecule HER antagonists; HER tyrosine kinase inhibitors; HER2 and EGFR dual tyrosine kinase inhibitors such as lapatinib/GW572016; antisense molecules (see, for example, WO2004/87207); and/or agents that bind to, or interfere with function of, downstream signaling molecules, such as MAPK or Akt (see FIG. **5**). Preferably, the HER inhibitor is an antibody or small molecule which binds to a HER receptor.

A "HER dimerization inhibitor" is an agent which inhibits formation of a HER dimer or HER heterodimer. Preferably, the HER dimerization inhibitor is an antibody, for example an antibody which binds to HER2 at the heterodimeric binding site thereof. The most preferred HER dimerization inhibitor herein is pertuzumab or MAb 2C4. Binding of 2C4 to the heterodimeric binding site of HER2 is illustrated in FIG. **4**. Other examples of HER dimerization inhibitors include antibodies which bind to EGFR and inhibit dimerization thereof with one or more other HER receptors (for example EGFR monoclonal antibody 806, MAb 806, which binds to activated or "untethered" EGFR; see Johns et al., *J. Biol. Chem.* 279(29):30375-30384 (2004)); antibodies which bind to HER3 and inhibit dimerization thereof with one or more other HER receptors; antibodies which bind to HER4 and inhibit dimerization thereof with one or more other HER receptors; peptide dimerization inhibitors (U.S. Pat. No. 6,417,168); antisense dimerization inhibitors; etc.

A "HER2 dimerization inhibitor" is an agent that inhibits formation of a dimer or heterodimer comprising HER2.

A "HER antibody" is an antibody that binds to a HER receptor. Optionally, the HER antibody further interferes with HER activation or function. Preferably, the HER antibody binds to the HER2 receptor. A HER2 antibody of particular interest herein is pertuzumab. Another example of a HER2 antibody is trastuzumab. Examples of EGFR antibodies include cetuximab and ABX0303.

"HER activation" refers to activation, or phosphorylation, of any one or more HER receptors. Generally, HER activation results in signal transduction (e.g. that caused by an intracellular kinase domain of a HER receptor phosphorylating tyrosine residues in the HER receptor or a substrate polypeptide). HER activation may be mediated by HER ligand binding to a HER dimer comprising the HER receptor of interest. HER ligand binding to a HER dimer may activate a kinase domain of one or more of the HER receptors in the dimer and thereby results in phosphorylation of tyrosine residues in one or more of the HER receptors and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s), such as Akt or MAPK intracellular kinases, see, FIG. **5**, for example.

"Phosphorylation" refers to the addition of one or more phosphate group(s) to a protein, such as a HER receptor, or substrate thereof.

An antibody which "inhibits HER dimerization" is an antibody which inhibits, or interferes with, formation of a HER dimer. Preferably, such an antibody binds to HER2 at the heterodimeric binding site thereof. The most preferred dimerization inhibiting antibody herein is pertuzumab or MAb 2C4. Binding of 2C4 to the heterodimeric binding site of HER2 is illustrated in FIG. **4**. Other examples of antibodies which inhibit HER dimerization include antibodies which bind to EGFR and inhibit dimerization thereof with one or more other HER receptors (for example EGFR monoclonal antibody 806, MAb 806, which binds to activated or "untethered" EGFR; see Johns et al., *J. Biol. Chem.* 279(29):30375-30384 (2004)); antibodies which bind to HER3 and inhibit dimerization thereof with one or more other HER receptors; and antibodies which bind to HER4 and inhibit dimerization thereof with one or more other HER receptors.

An antibody which "blocks ligand activation of a HER receptor more effectively than trastuzumab" is one which reduces or eliminates HER ligand activation of HER receptor(s) or HER dimer(s) more effectively (for example at least about 2-fold more effectively) than trastuzumab. Preferably, such an antibody blocks HER ligand activation of a HER receptor at least about as effectively as murine monoclonal antibody 2C4 or a Fab fragment thereof, or as pertuzumab or a Fab fragment thereof. One can evaluate the ability of an antibody to block ligand activation of a HER receptor by studying HER dimers directly, or by evaluating HER activation, or downstream signaling, which results from HER dimerization, and/or by evaluating the antibody-HER2 binding site, etc. Assays for screening for antibodies with the ability to inhibit ligand activation of a HER receptor more effectively than trastuzumab are described in Agus et al. *Cancer Cell* 2: 127-137 (2002) and WO01/00245 (Adams et al.). By way of example only, one may assay for: inhibition of HER dimer formation (see, e.g., FIG. 1A-B of Agus et al.

Cancer Cell 2: 127-137 (2002); and WO01/00245); reduction in HER ligand activation of cells which express HER dimers (WO01/00245 and FIG. 2A-B of Agus et al. *Cancer Cell* 2: 127-137 (2002), for example); blocking of HER ligand binding to cells which express HER dimers (WO01/00245, and FIG. 2E of Agus et al. *Cancer Cell* 2: 127-137 (2002), for example); cell growth inhibition of cancer cells (e.g. MCF7, MDA-MD-134, ZR-75-1, MD-MB-175, T-47D cells) which express HER dimers in the presence (or absence) of HER ligand (WO01/00245 and FIGS. 3A-D of Agus et al. *Cancer Cell* 2: 127-137 (2002), for instance); inhibition of downstream signaling (for instance, inhibition of HRG-dependent AKT phosphorylation or inhibition of HRG- or TGFα-dependent MAPK phosphorylation) (see, WO01/00245, and FIG. 2C-D of Agus et al. *Cancer Cell* 2: 127-137 (2002), for example). One may also assess whether the antibody inhibits HER dimerization by studying the antibody-HER2 binding site, for instance, by evaluating a structure or model, such as a crystal structure, of the antibody bound to HER2 (See, for example, Franklin et al. *Cancer Cell* 5:317-328 (2004)).

A "heterodimeric binding site" on HER2, refers to a region in the extracellular domain of HER2 that contacts, or interfaces with, a region in the extracellular domain of EGFR, HER3 or HER4 upon formation of a dimer therewith. The region is found in Domain II of HER2. Franklin et al. *Cancer Cell* 5:317-328 (2004).

The HER2 antibody may "inhibit HRG-dependent AKT phosphorylation" and/or inhibit "HRG- or TGFα-dependent MAPK phosphorylation" more effectively (for instance at least 2-fold more effectively) than trastuzumab (see Agus et al. *Cancer Cell* 2: 127-137 (2002) and WO01/00245, by way of example).

The HER2 antibody may be one which, like pertuzumab, does "not inhibit HER2 ectodomain cleavage" (Molina et al. *Cancer Res.* 61:4744-4749 (2001)). Trastuzumab, on the other hand, can inhibit HER2 ectodomain cleavage.

A HER2 antibody that "binds to a heterodimeric binding site" of HER2, binds to residues in domain II (and optionally also binds to residues in other of the domains of the HER2 extracellular domain, such as domains I and III), and can sterically hinder, at least to some extent, formation of a HER2-EGFR, HER2-HER3, or HER2-HER4 heterodimer. Franklin et al. *Cancer Cell* 5:317-328 (2004) characterize the HER2-pertuzumab crystal structure, deposited with the RCSB Protein Data Bank (ID Code IS78), illustrating an exemplary antibody that binds to the heterodimeric binding site of HER2.

An antibody that "binds to domain II" of HER2 binds to residues in domain II and optionally residues in other domain(s) of HER2, such as domains I and III. Preferably the antibody that binds to domain II binds to the junction between domains I, II and III of HER2.

Protein "expression" refers to conversion of the information encoded in a gene into messenger RNA (mRNA) and then to the protein.

Herein, a sample or cell that "expresses" a protein of interest (such as a HER receptor or HER ligand) is one in which mRNA encoding the protein, or the protein, including fragments thereof, is determined to be present in the sample or cell.

The technique of "polymerase chain reaction" or "PCR" as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Pat. No. 4,683,195 issued 28 Jul. 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.,* 51: 263 (1987); Erlich, ed., PCR Technology, (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample, comprising the use of a known nucleic acid (DNA or RNA) as a primer and utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

"Quantitative real time polymerase chain reaction" or "qRT-PCR" refers to a form of PCR wherein the amount of PCR product is measured at each step in a PCR reaction. This technique has been described in various publications including Cronin et al., *Am. J. Pathol.* 164(1):35-42 (2004); and Ma et al., *Cancer Cell* 5:607-616 (2004).

The term "microarray" refers to an ordered arrangement of hybridizable array elements, preferably polynucleotide probes, on a substrate.

The term "polynucleotide," when used in singular or plural, generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. The term "polynucleotide" specifically includes cDNAs. The term includes DNAs (including cDNAs) and RNAs that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritiated bases, are included within the term "polynucleotides" as defined herein. In general, the term "polynucleotide" embraces all chemically, enzymatically and/or metabolically modified forms of unmodified polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

The term "oligonucleotide" refers to a relatively short polynucleotide, including, without limitation, single-stranded deoxyribonucleotides, single- or double-stranded ribonucleotides, RNA:DNA hybrids and double-stranded DNAs. Oligonucleotides, such as single-stranded DNA probe oligonucleotides, are often synthesized by chemical methods, for example using automated oligonucleotide synthesizers that are commercially available. However, oligonucleotides

can be made by a variety of other methods, including in vitro recombinant DNA-mediated techniques and by expression of DNAs in cells and organisms.

The phrase "gene amplification" refers to a process by which multiple copies of a gene or gene fragment are formed in a particular cell or cell line. The duplicated region (a stretch of amplified DNA) is often referred to as "amplicon." Usually, the amount of the messenger RNA (mRNA) produced also increases in the proportion of the number of copies made of the particular gene expressed.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience Publishers, (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, typically: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50° C.; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42° C.; or (3) employ 50% formamide, 5×SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5×Denhardt's solution, sonicated salmon sperm DNA (50 &gr; g/ml), 0.1% SDS, and 10% dextran sulfate at 42° C., with washes at 42° C. in 0.2×SSC (sodium chloride/sodium citrate) and 50% formamide at 55° C., followed by a high-stringency wash consisting of 0.1×SSC containing EDTA at 55° C.

"Moderately stringent conditions" may be identified as described by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and % SDS) less stringent that those described above. An example of moderately stringent conditions is overnight incubation at 37° C. in a solution comprising: 20% formamide, 5×SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5×Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1×SSC at about 37-50° C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

A "native sequence" polypeptide is one which has the same amino acid sequence as a polypeptide (e.g., HER receptor or HER ligand) derived from nature, including naturally occurring or allelic variants. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope(s), except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. Such monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones or recombinant DNA clones. It should be understood that the selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, the monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler et al., *Nature,* 256:495 (1975); Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas 563-681, (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage display technologies (see, e.g., Clackson et al., *Nature,* 352:624-628 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 (1991); Sidhu et al., *J. Mol. Biol.* 338(2):299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5):1073-1093 (2004); Fellouse, *Proc. Nat. Acad. Sci. USA* 101(34):12467-12472 (2004); and Lee et al. *J. Immunol. Methods* 284(1-2):119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggemann et al., *Year in Immuno.,* 7:33 (1993); U.S. Pat. Nos. 5,545,806; 5,569,825; 5,591,669 (all of GenPharm); U.S. Pat. No. 5,545,807; WO 1997/17852; U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks et al., *Bio/Technology,* 10: 779-783 (1992); Lonberg et al., *Nature,* 368: 856-859 (1994); Morrison, *Nature,* 368: 812-813 (1994);

Fishwild et al., *Nature Biotechnology,* 14: 845-851 (1996); Neuberger, *Nature Biotechnology,* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.,* 13: 65-93 (1995)).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, Ape etc) and human constant region sequences, as well as "humanized" antibodies.

"Humanized" forms of non-human (e.g., rodent) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op, Struct. Biol.* 2:593-596 (1992).

Humanized HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 or trastuzumab (HERCEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337 expressly incorporated herein by reference; humanized 520C9 (WO93/21319); and humanized 2C4 antibodies such as pertuzumab as described herein.

For the purposes herein, "trastuzumab," "HERCEPTIN®," and "huMAb4D5-8" refer to an antibody comprising the light and heavy chain amino acid sequences in SEQ ID NOS. 15 and 16, respectively.

Herein, "pertuzumab" and "OMNITARG™" refer to an antibody comprising the light and heavy chain amino acid sequences in SEQ ID NOS. 13 and 14, respectively.

Differences between trastuzumab and pertuzumab functions are illustrated in FIG. **6**.

An "intact antibody" herein is one which comprises two antigen binding regions, and an Fc region. Preferably, the intact antibody has a functional Fc region.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragment(s).

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This

region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue.

Unless indicated otherwise, herein the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991), expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

A "functional Fc region" possesses an "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays as herein disclosed, for example.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, preferably one or more amino acid substitution(s). Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% homology with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and most preferably at least about 90% homology therewith, more preferably at least about 95% homology therewith.

Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes". There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS* (*USA*) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immu-*

nomethods 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)), and regulates homeostasis of immunoglobulins.

"Complement dependent cytotoxicity" or "CDC" refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (e.g. an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994). HER2 antibody scFv fragments are described in WO93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a variable heavy domain (V_H) connected to a variable light domain (V_L) in the same polypeptide chain (V_H–V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

A "naked antibody" is an antibody that is not conjugated to a heterologous molecule, such as a cytotoxic moiety or radiolabel.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

An "affinity matured" antibody is one with one or more alterations in one or more hypervariable regions thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. *Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. *Proc Nat. Acad. Sci*, USA 91:3809-3813 (1994); Schier et al. *Gene* 169:147-155 (1995); Yelton et al. *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al, *J. Mol. Biol.* 226:889-896 (1992).

The term "main species antibody" herein refers to the antibody structure in a composition which is the quantitatively predominant antibody molecule in the composition. In one embodiment, the main species antibody is a HER2 antibody, such as an antibody that binds to Domain II of HER2, antibody that inhibits HER dimerization more effectively than trastuzumab, and/or an antibody which binds to a heterodimeric binding site of HER2. The preferred embodiment herein of the main species antibody is one comprising the variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4, and most preferably comprising the light chain and heavy chain amino acid sequences in SEQ ID Nos. 13 and 14 (pertuzumab).

An "amino acid sequence variant" antibody herein is an antibody with an amino acid sequence which differs from a main species antibody. Ordinarily, amino acid sequence variants will possess at least about 70% homology with the main species antibody, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with the main species antibody. The amino acid sequence variants possess substitutions, deletions, and/or additions at certain positions within or adjacent to the amino acid sequence of the main species antibody. Examples of amino acid sequence variants herein include an acidic variant (e.g. deamidated antibody variant), a basic variant, an antibody with an amino-terminal leader extension (e.g. VHS—) on one or two light chains thereof, an antibody with a C-terminal lysine residue on one or two heavy chains thereof, etc, and includes combinations of variations to the amino acid sequences of heavy and/or light chains. The antibody variant of particular interest herein is the antibody comprising an amino-terminal leader extension on one or two light chains thereof, optionally further comprising other amino acid sequence and/or glycosylation differences relative to the main species antibody.

A "glycosylation variant" antibody herein is an antibody with one or more carbohydrate moeities attached thereto which differ from one or more carbohydrate moieties attached to a main species antibody. Examples of glycosylation variants herein include antibody with a G1 or G2 oligosaccharide structure, instead a G0 oligosaccharide structure, attached to an Fc region thereof, antibody with one or two carbohydrate moieties attached to one or two light chains thereof, antibody with no carbohydrate attached to one or two heavy chains of the antibody, etc, and combinations of glycosylation alterations.

Where the antibody has an Fc region, an oligosaccharide structure may be attached to one or two heavy chains of the antibody, e.g. at residue 299 (298, Eu numbering of residues). For pertuzumab, G0 was the predominant oligosaccharide structure, with other oligosaccharide structures such as G0-F, G-1, Man5, Man6, G1-1, G1(1-6), G1(1-3) and G2 being found in lesser amounts in the pertuzumab composition.

Unless indicated otherwise, a "G1 oligosaccharide structure" herein includes G-1, G1-1, G1(1-6) and G1(1-3) structures.

An "amino-terminal leader extension" herein refers to one or more amino acid residues of the amino-terminal leader sequence that are present at the amino-terminus of any one or more heavy or light chains of an antibody. An exemplary

amino-terminal leader extension comprises or consists of three amino acid residues, VHS, present on one or both light chains of an antibody variant.

A "deamidated" antibody is one in which one or more asparagine residues thereof has been derivitized, e.g. to an aspartic acid, a succinimide, or an iso-aspartic acid.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma (including medulloblastoma and retinoblastoma), sarcoma (including liposarcoma and synovial cell sarcoma), neuroendocrine tumors (including carcinoid tumors, gastrinoma, and islet cell cancer), mesothelioma, schwannoma (including acoustic neuroma), meningioma, adenocarcinoma, melanoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer (including metastatic breast cancer), colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, testicular cancer, esophagael cancer, tumors of the biliary tract, as well as head and neck cancer.

An "advanced" cancer is one which has spread outside the site or organ of origin, either by local invasion or metastasis.

A "refractory" cancer is one which progresses even though an anti-tumor agent, such as a chemotherapeutic agent, is being administered to the cancer patient. An example of a refractory cancer is one which is platinum refractory.

A "recurrent" cancer is one which has regrown, either at the initial site or at a distant site, after a response to initial therapy.

Herein, a "patient" is a human patient. The patient may be a "cancer patient," i.e. one who is suffering or at risk for suffering from one or more symptoms of cancer.

A "tumor sample" herein is a sample derived from, or comprising tumor cells from, a patient's tumor. Examples of tumor samples herein include, but are not limited to, tumor biopsies, circulating tumor cells, circulating plasma proteins, ascitic fluid, primary cell cultures or cell lines derived from tumors or exhibiting tumor-like properties, as well as preserved tumor samples, such as formalin-fixed, paraffin-embedded tumor samples or frozen tumor samples.

A "fixed" tumor sample is one which has been histologically preserved using a fixative.

A "formalin-fixed" tumor sample is one which has been preserved using formaldehyde as the fixative.

An "embedded" tumor sample is one surrounded by a firm and generally hard medium such as paraffin, wax, celloidin, or a resin. Embedding makes possible the cutting of thin sections for microscopic examination or for generation of tissue microarrays (TMAs).

A "paraffin-embedded" tumor sample is one surrounded by a purified mixture of solid hydrocarbons derived from petroleum.

Herein, a "frozen" tumor sample refers to a tumor sample which is, or has been, frozen.

A cancer or biological sample which "displays HER expression, amplification, or activation" is one which, in a diagnostic test, expresses (including overexpresses) a HER receptor, has amplified HER gene, and/or otherwise demonstrates activation or phosphorylation of a HER receptor.

A cancer or biological sample which "displays HER activation" is one which, in a diagnostic test, demonstrates activation or phosphorylation of a HER receptor. Such activation can be determined directly (e.g. by measuring HER phosphorylation by ELISA) or indirectly (e.g. by gene expression profiling or by detecting HER heterodimers, as described herein).

Herein, "gene expression profiling" refers to an evaluation of expression of one or more genes as a surrogate for determining HER phosphorylation directly.

A "phospho-ELISA assay" herein is an assay in which phosphorylation of one or more HER receptors, especially HER2, is evaluated in an enzyme-linked immunosorbent assay (ELISA) using a reagent, usually an antibody, to detect phosphorylated HER receptor, substrate, or downstream signaling molecule. Preferably, an antibody which detects phosphorylated HER2 is used. The assay may be performed on cell lysates, preferably from fresh or frozen biological samples.

A cancer cell with "HER receptor overexpression or amplification" is one which has significantly higher levels of a HER receptor protein or gene compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. HER receptor overexpression or amplification may be determined in a diagnostic or prognostic assay by evaluating increased levels of the HER protein present on the surface of a cell (e.g. via an immunohistochemistry assay; IHC). Alternatively, or additionally, one may measure levels of HER-encoding nucleic acid in the cell, e.g. via fluorescent in situ hybridization (FISH; see WO98/45479 published October, 1998), southern blotting, or polymerase chain reaction (PCR) techniques, such as quantitative real time PCR (qRT-PCR). One may also study HER receptor overexpression or amplification by measuring shed antigen (e.g., HER extracellular domain) in a biological fluid such as serum (see, e.g., U.S. Pat. No. 4,933,294 issued Jun. 12, 1990; WO91/05264 published Apr. 18, 1991; U.S. Pat. No. 5,401,638 issued Mar. 28, 1995; and Sias et al. *J. Immunol. Methods* 132: 73-80 (1990)). Aside from the above assays, various in vivo assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, e.g. a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, e.g. by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

Conversely, a cancer which "does not overexpress or amplify HER receptor" is one which does not have higher than normal levels of HER receptor protein or gene compared to a noncancerous cell of the same tissue type. Antibodies that inhibit HER dimerization, such as pertuzumab, may be used to treat cancer which does not overexpress or amplify HER2 receptor.

Herein, an "anti-tumor agent" refers to a drug used to treat cancer. Non-limiting examples of anti-tumor agents herein include chemotherapeutic agents, HER dimerization inhibitors, HER antibodies, antibodies directed against tumor associated antigens, anti-hormonal compounds, cytokines, EGFR-targeted drugs, anti-angiogenic agents, tyrosine kinase inhibitors, growth inhibitory agents and antibodies, cytotoxic agents, antibodies that induce apoptosis, COX inhibitors, farnesyl transferase inhibitors, antibodies that binds oncofetal protein CA 125, HER2 vaccines, Raf or ras inhibitors, liposomal doxorubicin, topotecan, taxane, dual

tyrosine kinase inhibitors, TLK286, EMD-7200, pertuzumab, trastuzumab, erlotinib, and bevacizumab.

An "approved anti-tumor agent" is a drug used to treat cancer which has been accorded marketing approval by a regulatory authority such as the Food and Drug Administration (FDA) or foreign equivalent thereof.

Where a HER dimerization inhibitor is administered as a "single anti-tumor agent" it is the only anti-tumor agent administered to treat the cancer, i.e. it is not administered in combination with another anti-tumor agent, such as chemotherapy.

By "standard of care" herein is intended the anti-tumor agent or agents that are routinely used to treat a particular form of cancer. For example, for platinum-resistant ovarian cancer, the standard of care is topotecan or liposomal doxorubicin.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially a HER expressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of HER expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13.

Examples of "growth inhibitory" antibodies are those which bind to HER2 and inhibit the growth of cancer cells overexpressing HER2. Preferred growth inhibitory HER2 antibodies inhibit growth of SK-BR-3 breast tumor cells in cell culture by greater than 20%, and preferably greater than 50% (e.g. from about 50% to about 100%) at an antibody concentration of about 0.5 to 30 μg/ml, where the growth inhibition is determined six days after exposure of the SK-BR-3 cells to the antibody (see U.S. Pat. No. 5,677,171 issued Oct. 14, 1997). The SK-BR-3 cell growth inhibition assay is described in more detail in that patent and hereinbelow. The preferred growth inhibitory antibody is a humanized variant of murine monoclonal antibody 4D5, e.g., trastuzumab.

An antibody which "induces apoptosis" is one which induces programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which overexpresses the HER2 receptor. Preferably the cell is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SK-BR-3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an annexin binding assay using BT474 cells (see below). Examples of HER2 antibodies that induce apoptosis are 7C2 and 7F3.

The "epitope 2C4" is the region in the extracellular domain of HER2 to which the antibody 2C4 binds. In order to screen for antibodies which bind to the 2C4 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Preferably the antibody blocks 2C4's binding to HER2 by about 50% or more. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 2C4 epitope of HER2. Epitope 2C4 comprises residues from Domain II in the extracellular domain of HER2. 2C4 and pertuzumab binds to the extracellular domain of HER2 at the junction of domains I, II and III. Franklin et al. *Cancer Cell* 5:317-328 (2004).

The "epitope 4D5" is the region in the extracellular domain of HER2 to which the antibody 4D5 (ATCC CRL 10463) and trastuzumab bind. This epitope is close to the transmembrane domain of HER2, and within Domain IV of HER2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of HER2 (e.g. any one or more residues in the region from about residue 529 to about residue 625, inclusive of the HER2 ECD, residue numbering including signal peptide).

The "epitope 7C2/7F3" is the region at the N terminus, within Domain I, of the extracellular domain of HER2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on HER2 (e.g. any one or more of residues in the region from about residue 22 to about residue 53 of the HER2 ECD, residue numbering including signal peptide).

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with cancer as well as those in which cancer is to be prevented. Hence, the patient to be treated herein may have been diagnosed as having cancer or may be predisposed or susceptible to cancer.

The term "effective amount" refers to an amount of a drug effective to treat cancer in the patient. The effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. The effective amount may extend progression free survival (e.g. as measured by Response Evaluation Criteria for Solid Tumors, RECIST, or CA-125 changes), result in an objective response (including a partial response, PR, or complete response, CR), increase overall survival time, and/or improve one or more symptoms of cancer (e.g. as assessed by FOR).

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or

causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At^{211}, I^{131}, I^{125}, Y^{90}, R^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; TLK 286 (TELCYTA™); acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; bisphosphonates, such as clodronate; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammall and calicheamicin omegal (see, e.g., *Agnew, Chem. Intl. Ed. Engl.,* 33: 183-186 (1994)) and anthracyclines such as annamycin, AD 32, alcarubicin, daunorubicin, dexrazoxane, DX-52-1, epirubicin, GPX-100, idarubicin, KRN5500, menogaril, dynemicin, including dynemicin A, an esperamicin, neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, liposomal doxorubicin, and deoxydoxorubicin), esorubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, and zorubicin; folic acid analogues such as denopterin, pteropterin, and trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, and floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, and testolactone; anti-adrenals such as aminoglutethimide, mitotane, and trilostane; folic acid replenisher such as folinic acid (leucovorin); aceglatone; anti-folate anti-neoplastic agents such as ALIMTA®, LY231514 pemetrexed, dihydrofolate reductase inhibitors such as methotrexate, anti-metabolites such as 5-fluorouracil (5-FU) and its prodrugs such as UFT, S-1 and capecitabine, and thymidylate synthase inhibitors and glycinamide ribonucleotide formyltransferase inhibitors such as raltitrexed (TOMUDEX™, TDX); inhibitors of dihydropyrimidine dehydrogenase such as eniluracil; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids and taxanes, e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Ill.), and TAXOTERE® docetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; platinum; platinum analogs or platinum-based analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine (VELBAM); etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); vinca alkaloid; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovorin.

Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON® toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; and pharmaceutically acceptable salts, acids or derivatives of any of the above. An "antimetabolite chemotherapeutic agent" is an agent which is structurally similar to a metabolite, but can not be used by the body in a productive manner. Many antimetabolite chemotherapeutic agents interfere with the production of the nucleic acids, RNA and DNA. Examples

of antimetabolite chemotherapeutic agents include gemcitabine (GEMZAR®), 5-fluorouracil (5-FU), capecitabine (XELODA™), 6-mercaptopurine, methotrexate, 6-thioguanine, pemetrexed, raltitrexed, arabinosylcytosine ARA-C cytarabine (CYTOSAR-U®), dacarbazine (DTIC-DOME®), azocytosine, deoxycytosine, pyridmidene, fludarabine (FLUDARA®), cladrabine, 2-deoxy-D-glucose etc. The preferred antimetabolite chemotherapeutic agent is gemcitabine.

"Gemcitabine" or "2'-deoxy-2',2'-difluorocytidine monohydrochloride (b-isomer)" is a nucleoside analogue that exhibits antitumor activity. The empirical formula for gemcitabine HCl is C9H11F2N3O4.HCl. Gemcitabine HCl is sold by Eli Lilly under the trademark GEMZAR®.

A "platinum-based chemotherapeutic agent" comprises an organic compound which contains platinum as an integral part of the molecule. Examples of platinum-based chemotherapeutic agents include carboplatin, cisplatin, and oxaliplatinum.

By "platinum-based chemotherapy" is intended therapy with one or more platinum-based chemotherapeutic agents, optionally in combination with one or more other chemotherapeutic agents.

By "chemotherapy-resistant" cancer is meant that the cancer patient has progressed while receiving a chemotherapy regimen (i.e. the patient is "chemotherapy refractory"), or the patient has progressed within 12 months (for instance, within 6 months) after completing a chemotherapy regimen.

By "platinum-resistant" cancer is meant that the cancer patient has progressed while receiving platinum-based chemotherapy (i.e. the patient is "platinum refractory"), or the patient has progressed within 12 months (for instance, within 6 months) after completing a platinum-based chemotherapy regimen.

An "anti-angiogenic agent" refers to a compound which blocks, or interferes with to some degree, the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or antibody that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. The preferred anti-angiogenic factor herein is an antibody that binds to vascular endothelial growth factor (VEGF), such as bevacizumab (AVASTIN®).

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

As used herein, the term "EGFR-targeted drug" refers to a therapeutic agent that binds to EGFR and, optionally, inhibits EGFR activation. Examples of such agents include antibodies and small molecules that bind to EGFR. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225 or Cetuximab; ERBUTIX®) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.); IMC-11F8, a fully human, EGFR-targeted antibody (Imclone); antibodies that bind type II mutant EGFR (U.S. Pat. No. 5,212,290); humanized and chimeric antibodies that bind EGFR as described in U.S. Pat. No. 5,891,996; and human antibodies that bind EGFR, such as ABX-EGF (see WO98/50433, Abgenix); EMD 55900 (Stragliotto et al. *Eur. J. Cancer* 32A:636-640 (1996)); EMD7200 (matuzumab) a humanized EGFR antibody directed against EGFR that competes with both EGF and TGF-alpha for EGFR binding; and mAb 806 or humanized mAb 806 (Johns et al., *J. Biol. Chem.* 279(29):30375-30384 (2004)). The anti-EGFR antibody may be conjugated with a cytotoxic agent, thus generating an immunoconjugate (see, e.g., EP659,439A2, Merck Patent GmbH). Examples of small molecules that bind to EGFR include ZD1839 or Gefitinib (IRESSA™; Astra Zeneca); CP-358774 or Erlotinib (TARCEVA™; Genentech/OSI); and AG1478, AG1571 (SU 5271; Sugen); EMD-7200.

A "tyrosine kinase inhibitor" is a molecule which inhibits tyrosine kinase activity of a tyrosine kinase such as a HER receptor. Examples of such inhibitors include the EGFR-targeted drugs noted in the preceding paragraph; small molecule HER2 tyrosine kinase inhibitor such as TAK165 available from Takeda; CP-724,714, an oral selective inhibitor of the ErbB2 receptor tyrosine kinase (Pfizer and OSI); dual-HER inhibitors such as EKB-569 (available from Wyeth) which preferentially binds EGFR but inhibits both HER2 and EGFR-overexpressing cells; GW572016 (available from Glaxo) an oral HER2 and EGFR tyrosine kinase inhibitor; PKI-166 (available from Novartis); pan-HER inhibitors such as canertinib (CI-1033; Pharmacia); Raf-1 inhibitors such as antisense agent ISIS-5132 available from ISIS Pharmaceuticals which inhibits Raf-1 signaling; non-HER targeted TK inhibitors such as Imatinib mesylate (Gleevac™) available from Glaxo; MAPK extracellular regulated kinase I inhibitor CI-1040 (available from Pharmacia); quinazolines, such as PD 153035,4-(3-chloroanilino) quinazoline; pyridopyrimidines; pyrimidopyrimidines; pyrrolopyrimidines, such as CGP 59326, CGP 60261 and CGP 62706; pyrazolopyrimidines, 4-(phenylamino)-7H-pyrrolo[2,3-d]pyrimidines; curcumin (diferuloyl methane, 4,5-bis(4-fluoroanilino)phthalimide); tyrphostines containing nitrothiophene moieties; PD-0183805 (Warner-Lamber); antisense molecules (e.g. those that bind to HER-encoding nucleic acid); quinoxalines (U.S. Pat. No. 5,804,396); tryphostins (U.S. Pat. No. 5,804,396); ZD6474 (Astra Zeneca); PTK-787 (Novartis/Schering AG); pan-HER inhibitors such as CI-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); Imatinib mesylate (Gleevac; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); CI-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Sugen); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-1C11 (Imclone); or as described in any of the following patent publications: U.S. Pat. No. 5,804,396; WO99/09016 (American Cyanimid); WO98/43960 (American Cyanamid); WO97/38983 (Warner Lambert); WO99/06378 (Warner

Lambert); WO99/06396 (Warner Lambert); WO96/30347 (Pfizer, Inc); WO96/33978 (Zeneca); WO96/3397 (Zeneca); and WO96/33980 (Zeneca).

A "fixed" or "flat" dose of a therapeutic agent herein refers to a dose that is administered to a human patient without regard for the weight (WT) or body surface area (BSA) of the patient. The fixed or flat dose is therefore not provided as a mg/kg dose or a mg/m^2 dose, but rather as an absolute amount of the therapeutic agent.

A "loading" dose herein generally comprises an initial dose of a therapeutic agent administered to a patient, and is followed by one or more maintenance dose(s) thereof. Generally, a single loading dose is administered, but multiple loading doses are contemplated herein. Usually, the amount of loading dose(s) administered exceeds the amount of the maintenance dose(s) administered and/or the loading dose(s) are administered more frequently than the maintenance dose(s), so as to achieve the desired steady-state concentration of the therapeutic agent earlier than can be achieved with the maintenance dose(s).

A "maintenance" dose herein refers to one or more doses of a therapeutic agent administered to the patient over a treatment period. Usually, the maintenance doses are administered at spaced treatment intervals, such as approximately every week, approximately every 2 weeks, approximately every 3 weeks, or approximately every 4 weeks.

II. Production of Antibodies

Since, in the preferred embodiment, the HER dimerization inhibitor is an antibody, a description follows as to exemplary techniques for the production of HER antibodies used in accordance with the present invention. The HER antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of a HER receptor or a portion thereof, containing the desired epitope. Alternatively, cells expressing HER at their cell surface (e.g. NIH-3T3 cells transformed to overexpress HER2; or a carcinoma cell line such as SK-BR-3 cells, see Stancovski et al. *PNAS* (*USA*) 88:8691-8695 (1991)) can be used to generate antibodies. Other forms of HER receptor useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Various methods for making monoclonal antibodies herein are available in the art. For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); and Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such

as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130: 151-188 (1992).

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352: 624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy chain and light chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; and Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized Antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

WO01/00245 describes production of exemplary humanized HER2 antibodies which bind HER2 and block ligand activation of a HER receptor. The humanized antibody of particular interest herein blocks EGF, TGF-α and/or HRG mediated activation of MAPK essentially as effectively as murine monoclonal antibody 2C4 (or a Fab fragment thereof) and/or binds HER2 essentially as effectively as murine monoclonal antibody 2C4 (or a Fab fragment thereof). The humanized antibody herein may, for example, comprise nonhuman hypervariable region residues incorporated into a human variable heavy domain and may further comprise a framework region (FR) substitution at a position selected from the group consisting of 69H, 71H and 73H utilizing the variable domain numbering system set forth in Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). In one embodiment, the humanized antibody comprises FR substitutions at two or all of positions 69H, 71H and 73H.

An exemplary humanized antibody of interest herein comprises variable heavy domain complementarity determining residues GFTFTDYTMX, where X is preferrably D or S (SEQ ID NO:7); DVNPNSGGSIYNQRFKG (SEQ ID NO:8); and/or NLGPSFYFDY (SEQ ID NO:9), optionally comprising amino acid modifications of those CDR residues, e.g. where the modifications essentially maintain or improve affinity of the antibody. For example, the antibody variant of interest may have from about one to about seven or about five amino acid substitutions in the above variable heavy CDR sequences. Such antibody variants may be prepared by affinity maturation, e.g., as described below. The most preferred

humanized antibody comprises the variable heavy domain amino acid sequence in SEQ ID NO:4.

The humanized antibody may comprise variable light domain complementarity determining residues KASQDVSIGVA (SEQ ID NO:10); SASYX^{1}X^{2}X^3, where X^1 is preferably R or L, X^2 is preferably Y or E, and X^3 is preferably T or S (SEQ ID NO:11); and/or QQYYIYPYT (SEQ ID NO:12), e.g. in addition to those variable heavy domain CDR residues in the preceding paragraph. Such humanized antibodies optionally comprise amino acid modifications of the above CDR residues, e.g. where the modifications essentially maintain or improve affinity of the antibody. For example, the antibody variant of interest may have from about one to about seven or about five amino acid substitutions in the above variable light CDR sequences. Such antibody variants may be prepared by affinity maturation, e.g., as described below. The most preferred humanized antibody comprises the variable light domain amino acid sequence in SEQ ID NO:3.

The present application also contemplates affinity matured antibodies which bind HER2 and block ligand activation of a HER receptor. The parent antibody may be a human antibody or a humanized antibody, e.g., one comprising the variable light and/or variable heavy sequences of SEQ ID Nos. 3 and 4, respectively (i.e. comprising the VL and/or VH of pertuzumab). The affinity matured antibody preferably binds to HER2 receptor with an affinity superior to that of murine 2C4 or pertuzumab (e.g. from about two or about four fold, to about 100 fold or about 1000 fold improved affinity, e.g. as assessed using a HER2-extracellular domain (ECD) ELISA). Exemplary variable heavy CDR residues for substitution include H28, H30, H34, H35, H64, H96, H99, or combinations of two or more (e.g. two, three, four, five, six, or seven of these residues). Examples of variable light CDR residues for alteration include L28, L50, L53, L56, L91, L92, L93, L94, L96, L97 or combinations of two or more (e.g. two to three, four, five or up to about ten of these residues).

Various forms of the humanized antibody or affinity matured antibody are contemplated. For example, the humanized antibody or affinity matured antibody may be an antibody fragment, such as a Fab, which is optionally conjugated with one or more cytotoxic agent(s) in order to generate an immunoconjugate. Alternatively, the humanized antibody or affinity matured antibody may be an intact antibody, such as an intact IgG1 antibody. The preferred intact IgG1 antibody comprises the light chain sequence in SEQ ID NO:13 and the heavy chain sequence in SEQ ID NO:14.

(iv) Human Antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germline mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and U.S. Pat. Nos. 5,591,669, 5,589,369 and 5,545,807. Alternatively, phage display technology (McCafferty et al., *Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S, and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., *Nature,* 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., *J. Mol. Biol.* 222:581-597 (1991), or Griffith et al., *EMBO J.* 12:725-734 (1993). See, also, U.S. Pat. Nos. 5,565,332 and 5,573,905.

As discussed above, human antibodies may also be generated by in vitro activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

Human HER2 antibodies are described in U.S. Pat. No. 5,772,997 issued Jun. 30, 1998 and WO 97/00271 published Jan. 3, 1997.

(v) Antibody Fragments

Various techniques have been developed for the production of antibody fragments comprising one or more antigen binding regions. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')$_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, F(ab')$_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the HER2 protein. Other such antibodies may combine a HER2 binding site with binding site(s) for EGFR, HER3 and/or HER4. Alternatively, a HER2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the HER2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express HER2. These antibodies possess a HER2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin,

anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

WO 96/16673 describes a bispecific HER2/FcγRIII antibody and U.S. Pat. No. 5,837,234 discloses a bispecific HER2/FcγRI antibody IDM1 (Osidem). A bispecific HER2/Fcα antibody is shown in WO98/02463. U.S. Pat. No. 5,821,337 teaches a bispecific HER2/CD3 antibody. MDX-210 is a bispecific HER2-FcγRIII Ab.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in U.S. Pat. No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

(vii) Other Amino Acid Sequence Modifications

Amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells *Science,* 244:1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed antibody variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys

TABLE 1-continued

Original Residue	Exemplary Substitutions	Preferred Substitutions
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in *Biochemistry*, second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His (H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g., a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monova-

lent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human HER2. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that lacks fucose attached to an Fc region of the antibody are described in U.S. Pat Appl No US 2003/0157108 A1, Presta, L. See also US 2004/0093621 A1 (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetylglucosamine (GlcNAc) in the carbohydrate attached to an Fc region of the antibody are referenced in WO03/011878, Jean-Mairet et al. *and U.S. Pat. No.* 6,602,684, Umana et al. Antibodies with at least one galactose residue in the oligosaccharide attached to an Fc region of the antibody are reported in WO97/30087, Patel et al. See, also, WO98/58964 (Raju, S.) and WO99/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof.

It may be desirable to modify the antibody of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-(1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional crosslinkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

WO00/42072 (Presta, L.) describes antibodies with improved ADCC function in the presence of human effector cells, where the antibodies comprise amino acid substitutions in the Fc region thereof. Preferably, the antibody with improved ADCC comprises substitutions at positions 298, 333, and/or 334 of the Fe region (Eu numbering of residues). Preferably the altered Fc region is a human IgG1 Fc region comprising or consisting of substitutions at one, two or three of these positions. Such substitutions are optionally combined with substitution(s) which increase C1q binding and/or CDC.

Antibodies with altered C1q binding and/or complement dependent cytotoxicity (CDC) are described in WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.). The antibodies comprise an amino acid substitution at one or more of amino acid positions 270, 322, 326, 327, 329, 313, 333 and/or 334 of the Fc region thereof (Eu numbering of residues).

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Pat. No. 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

Antibodies with improved binding to the neonatal Fc receptor (FcRn), and increased half-lives, are described in WO00/42072 (Presta, L.) and US2005/0014934A1 (Hinton et al.). These antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. For example, the Fc region may have substitutions at one or more of positions 238, 250, 256, 265, 272, 286, 303, 305, 307, 311, 312, 314, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424, 428 or 434 (Eu numbering of residues). The preferred Fc region-comprising antibody variant with improved FcRn binding comprises amino acid substitutions at one, two or three of positions 307, 380 and 434 of the Fc region thereof (Eu numbering of residues).

Engineered antibodies with three or more (preferably four) functional antigen binding sites are also contemplated (U.S. Appln No. US2002/0004587 A1, Miller et al.).

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

(viii) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. One may further select antibodies with certain biological characteristics, as desired.

To identify an antibody which blocks ligand activation of a HER receptor, the ability of the antibody to block HER ligand binding to cells expressing the HER receptor (e.g. in conju-

gation with another HER receptor with which the HER receptor of interest forms a HER hetero-oligomer) may be determined. For example, cells naturally expressing, or transfected to express, HER receptors of the HER hetero-oligomer may be incubated with the antibody and then exposed to labeled HER ligand. The ability of the antibody to block ligand binding to the HER receptor in the HER hetero-oligomer may then be evaluated.

For example, inhibition of HRG binding to MCF7 breast tumor cell lines by HER2 antibodies may be performed using monolayer MCF7 cultures on ice in a 24-well-plate format essentially as described in WO01/00245. HER2 monoclonal antibodies may be added to each well and incubated for 30 minutes. ^{125}I-labeled rHRGβ1$_{177-224}$ (25 μm) may then be added, and the incubation may be continued for 4 to 16 hours. Dose response curves may be prepared and an IC_{50} value may be calculated for the antibody of interest. In one embodiment, the antibody which blocks ligand activation of a HER receptor will have an IC_{50} for inhibiting HRG binding to MCF7 cells in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG binding to MCF7 cells in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

Alternatively, or additionally, the ability of an antibody to block HER ligand-stimulated tyrosine phosphorylation of a HER receptor present in a HER hetero-oligomer may be assessed. For example, cells endogenously expressing the HER receptors or transfected to expressed them may be incubated with the antibody and then assayed for HER ligand-dependent tyrosine phosphorylation activity using an anti-phosphotyrosine monoclonal (which is optionally conjugated with a detectable label). The kinase receptor activation assay described in U.S. Pat. No. 5,766,863 is also available for determining HER receptor activation and blocking of that activity by an antibody.

In one embodiment, one may screen for an antibody which inhibits HRG stimulation of p180 tyrosine phosphorylation in MCF7 cells essentially as described in WO01/00245. For example, the MCF7 cells may be plated in 24-well plates and monoclonal antibodies to HER2 may be added to each well and incubated for 30 minutes at room temperature; then rHRGβ1$_{177-244}$ may be added to each well to a final concentration of 0.2 nM, and the incubation may be continued for 8 minutes. Media may be aspirated from each well, and reactions may be stopped by the addition of 100 μl of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 μl) may be electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (at 1 μg/ml) immunoblots may be developed, and the intensity of the predominant reactive band at M_r~180,000 may be quantified by reflectance densitometry. The antibody selected will preferably significantly inhibit HRG stimulation of p180 tyrosine phosphorylation to about 0-35% of control in this assay. A dose-response curve for inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry may be prepared and an IC_{50} for the antibody of interest may be calculated. In one embodiment, the antibody which blocks ligand activation of a HER receptor will have an IC_{50} for inhibiting HRG stimulation of p180 tyrosine phosphorylation in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG stimulation of p180 tyrosine phosphorylation in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

One may also assess the growth inhibitory effects of the antibody on MDA-MB-175 cells, e.g., essentially as described in Schaefer et al. *Oncogene* 15:1385-1394 (1997). According to this assay, MDA-MB-175 cells may be treated with a HER2 monoclonal antibody (10 μg/mL) for 4 days and stained with crystal violet. Incubation with a HER2 antibody may show a growth inhibitory effect on this cell line similar to that displayed by monoclonal antibody 2C4. In a further embodiment, exogenous HRG will not significantly reverse this inhibition. Preferably, the antibody will be able to inhibit cell proliferation of MDA-MB-175 cells to a greater extent than monoclonal antibody 4D5 (and optionally to a greater extent than monoclonal antibody 7F3), both in the presence and absence of exogenous HRG.

In one embodiment, the HER2 antibody of interest may block heregulin dependent association of HER2 with HER3 in both MCF7 and SK-BR-3 cells as determined in a co-immunoprecipitation experiment such as that described in WO01/00245 substantially more effectively than monoclonal antibody 4D5, and preferably substantially more effectively than monoclonal antibody 7F3.

To identify growth inhibitory HER2 antibodies, one may screen for antibodies which inhibit the growth of cancer cells which overexpress HER2. In one embodiment, the growth inhibitory antibody of choice is able to inhibit growth of SK-BR-3 cells in cell culture by about 20-100% and preferably by about 50-100% at an antibody concentration of about 0.5 to 30 μg/ml. To identify such antibodies, the SK-BR-3 assay described in U.S. Pat. No. 5,677,171 can be performed. According to this assay, SK-BR-3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin streptomycin. The SK-BR-3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 0.5 to 30 μg/ml of the HER2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SK-BR-3 cells by about 20-100% or about 50-100% may be selected as growth inhibitory antibodies. See U.S. Pat. No. 5,677,171 for assays for screening for growth inhibitory antibodies, such as 4D5 and 3E8.

In order to select for antibodies which induce apoptosis, an annexin binding assay using BT474 cells is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the monoclonal antibody. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies. In addition to the annexin binding assay, a DNA staining assay using BT474 cells is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be

selected as pro-apoptotic antibodies using this assay. See WO98/17797 for assays for screening for antibodies which induce apoptosis, such as 7C2 and 7F3.

To screen for antibodies which bind to an epitope on HER2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed to assess whether the antibody cross-blocks binding of an antibody, such as 2C4 or pertuzumab, to HER2. Alternatively, or additionally, epitope mapping can be performed by methods known in the art and/or one can study the antibody-HER2 structure (Franklin et al. *Cancer Cell* 5:317-328 (2004)) to see what domain(s) of HER2 is/are bound by the antibody.

(ix) Pertuzumab Compositions

In one embodiment of a HER2 antibody composition, the composition comprises a mixture of a main species pertuzumab antibody and one or more variants thereof. The preferred embodiment herein of a pertuzumab main species antibody is one comprising the variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4, and most preferably comprising a light chain amino acid sequence selected from SEQ ID No. 13 and 17, and a heavy chain amino acid sequence selected from SEQ ID No. 14 and 18 (including deamidated and/or oxidized variants of those sequences). In one embodiment, the composition comprises a mixture of the main species pertuzumab antibody and an amino acid sequence variant thereof comprising an amino-terminal leader extension. Preferably, the amino-terminal leader extension is on a light chain of the antibody variant (e.g. on one or two light chains of the antibody variant). The main species HER2 antibody or the antibody variant may be an full length antibody or antibody fragment (e.g. Fab of F(ab')2 fragments), but preferably both are full length antibodies. The antibody variant herein may comprise an amino-terminal leader extension on any one or more of the heavy or light chains thereof. Preferably, the amino-terminal leader extension is on one or two light chains of the antibody. The amino-terminal leader extension preferably comprises or consists of VHS—. Presence of the amino-terminal leader extension in the composition can be detected by various analytical techniques including, but not limited to, N-terminal sequence analysis, assay for charge heterogeneity (for instance, cation exchange chromatography or capillary zone electrophoresis), mass spectrometry, etc. The amount of the antibody variant in the composition generally ranges from an amount that constitutes the detection limit of any assay (preferably N-terminal sequence analysis) used to detect the variant to an amount less than the amount of the main species antibody. Generally, about 20% or less (e.g. from about 1% to about 15%, for instance from 5% to about 15%) of the antibody molecules in the composition comprise an amino-terminal leader extension. Such percentage amounts are preferably determined using quantitative N-terminal sequence analysis or cation exchange analysis (preferably using a high-resolution, weak cation-exchange column, such as a PROPAC WCX-10™ cation exchange column). Aside from the amino-terminal leader extension variant, further amino acid sequence alterations of the main species antibody and/or variant are contemplated, including but not limited to an antibody comprising a C-terminal lysine residue on one or both heavy chains thereof, a deamidated antibody variant, etc.

Moreover, the main species antibody or variant may further comprise glycosylation variations, non-limiting examples of which include antibody comprising a G1 or G2 oligosaccharide structure attached to the Fc region thereof, antibody comprising a carbohydrate moiety attached to a light chain thereof (e.g. one or two carbohydrate moieties, such as glucose or galactose, attached to one or two light chains of the antibody, for instance attached to one or more lysine residues), antibody comprising one or two non-glycosylated heavy chains, or antibody comprising a sialidated oligosaccharide attached to one or two heavy chains thereof etc.

The composition may be recovered from a genetically engineered cell line, e.g. a Chinese Hamster Ovary (CHO) cell line expressing the HER2 antibody, or may be prepared by peptide synthesis.

(x) Immunoconjugates

The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. a small molecule toxin or an enzymatically active toxin of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, a maytansine (U.S. Pat. No. 5,208,020), a trichothene, and CC1065 are also contemplated herein.

In one preferred embodiment of the invention, the antibody is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody (Chari et al. *Cancer Research* 52: 127-131 (1992)) to generate a maytansinoid-antibody immunoconjugate.

Another immunoconjugate of interest comprises an antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ^I_1 (Hinman et al. *Cancer Research* 53: 3336-3342 (1993) and Lode et al. *Cancer Research* 58: 2925-2928 (1998)). See, also, U.S. Pat. Nos. 5,714,586; 5,712,374; 5,264,586; and 5,773,001 expressly incorporated herein by reference.

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published Oct. 28, 1993.

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

A variety of radioactive isotopes are available for the production of radioconjugated HER2 antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis(p-azido-

benzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al. *Cancer Research* 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis.

Other immunoconjugates are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences,* 16th edition, Oslo, A., Ed., (1980).

The antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA,* 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published Oct. 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19) 1484 (1989).

III. Selecting Patients for Therapy

The patient herein is optionally subjected to a diagnostic test prior to therapy. For example, the diagnostic test may evaluate HER (e.g. HER2 or EGFR) expression (including overexpression), amplification, and/or activation (including phosphorylation or dimerization).

Generally, if a diagnostic test is performed, a sample may be obtained from a patient in need of therapy. Where the subject has cancer, the sample is generally a tumor sample. In the preferred embodiment, the tumor sample is from an ovarian cancer, peritoneal cancer, fallopian tube cancer, metastatic breast cancer (MBC), non-small cell lung cancer (NSCLC), prostate cancer, or colorectal cancer tumor sample.

The biological sample herein may be a fixed sample, e.g. a formalin fixed, paraffin-embedded (FFPE) sample, or a frozen sample.

According to one embodiment of the invention herein, the patient selected for therapy has a tumor displaying HER (and preferably HER2) activation. In one embodiment, the extent of HER (or HER2) activation in cancer cells significantly exceeds the level of activation of that receptor in noncancerous cells of the same tissue type. Such excessive activation may result from overexpression of the HER receptor and/or greater than normal levels of a HER ligand available for activating the HER receptor in the cancer cells. Such excessive activation may cause and/or be caused by the malignant state of a cancer cell. In some embodiments, the cancer will be subjected to a diagnostic or prognostic assay to determine whether amplification and/or overexpression of a HER receptor is occurring which results in such excessive activation of the HER receptor. Alternatively, or additionally, the cancer may be subjected to a diagnostic or prognostic assay to determine whether amplification and/or overexpression a HER ligand is occurring in the cancer which attributes to excessive activation of the receptor. In a subset of such cancers, excessive activation of the receptor may result from an autocrine stimulatory pathway. Various assays for determining HER activation will be described in more detail below. The preferred methods for determining HER activation are: detecting the presence of HER dimers or heterodimers, evaluating HER or HER2 phosphorylation, and gene expression profiling.

(i) HER Dimers

Tumors samples can be assessed for the presence of HER dimers, as indicating HER or HER2 activation. Any method known in the art may be used to detect HER2 dimers, such as EGFR-HER2, HER2-HER3, in tumors. Several preferred methods are described below. These methods detect noncovalent protein-protein interactions or otherwise indicate proximity between proteins of interest.

Immunoaffinity-based methods, such as immunoprecipitation or ELISA, may be used to detect HER dimers. In one embodiment, HER2 antibodies are used to immunoprecipitate complexes comprising HER2 from tumor cells, and the resulting immunoprecipitant is then probed for the presence of EGFR or HER3 by immunoblotting. In another embodiment, EGFR or HER3 antibodies may be used for the immunoprecipitation step and the immunoprecipitant then probed with HER2 antibodies. In a further embodiment, HER ligands specific to EGFR, HER3, EGFR-HER2 complexes or HER2-HER3 complexes may be used to precipitate complexes, which are then probed for the presence of HER2. For example, ligands may be conjugated to avidin and complexes purified on a biotin column.

In other embodiments, such as ELISA or antibody "sandwich"-type assays, antibodies to HER2 are immobilized on a solid support, contacted with tumor cells or tumor cell lysate, washed, and then exposed to antibody against EGFR or HER3. Binding of the latter antibody, which may be detected directly or by a secondary antibody conjugated to a detectable label, indicates the presence of heterodimers. In certain embodiments, EGFR or HER3 antibody is immobilized, and HER2 antibody is used for the detection step. In other embodiments HER ligands may be used in place of, or in combination with HER antibodies.

Chemical or UV cross-linking may also be used to covalently join dimers on the surface of living cells. Examples of chemical cross-linkers include dithiobis(succinimidyl) propionate (DSP) and 3,3'dithiobis(sulphosuccinimidyl) propionate (DTSSP). In one embodiment, cell extracts from chemically cross-linked tumor cells are analyzed by

SDS-PAGE and immunoblotted with antibodies to EGFR and/or HER3. A supershifted band of the appropriate molecular weight most likely represents EGFR-HER2 or HER2-HER3 dimers, as HER2 is the preferred dimerization partner for EGFR and HER3. This result may be confirmed by subsequent immunoblotting with HER2 antibodies.

Fluorescence resonance energy transfer (FRET) may also be used to detect EGFR-HER2 or HER2-HER3 dimers. FRET detects protein conformational changes and protein-protein interactions in vivo and in vitro based on the transfer of energy from a donor fluorophore to an acceptor fluorophore. Selvin, *Nat. Struct. Biol.,* 7:730-34 (2000). Energy transfer takes place only if the donor fluorophore is in sufficient proximity to the acceptor fluorophore. In a typical FRET experiment, two proteins or two sites on a single protein are labeled with different fluorescent probes. One of the probes, the donor probe, is excited to a higher energy state by incident light of a specified wavelength. The donor probe then transmits its energy to the second probe, the acceptor probe, resulting in a reduction in the donor's fluorescence intensity and an increase in the acceptor's fluorescence emission. To measure the extent of energy transfer, the donor's intensity in a sample labeled with donor and acceptor probes is compared with its intensity in a sample labeled with donor probe only. Optionally, acceptor intensity is compared in donor/acceptor and acceptor only samples. Suitable probes are known in the art and include, for example, membrane permeant dyes, such as fluorescein and rhodamine, organic dyes, such as the cyanine dyes, and lanthanide atoms. Methods and instrumentation for detecting and measuring energy transfer are also known in the art.

FRET-based techniques suitable for detecting and measuring protein-protein interactions in individual cells are also known in the art. For example, donor photobleaching fluorescence resonance energy transfer (pbFRET) microscopy and fluorescence lifetime imaging microscopy (FLIM) may be used to detect the dimerization of cell surface receptors. Gadella & Jovin, *J. Cell Biol.,* 129:1543-58 (1995). In one embodiment, pbFRET is used on cells either "in suspension" or "in situ" to detect and measure the formation of EGFR-HER2 or HER2-HER3 dimers, as described in Nagy et al., *Cytometry,* 32:120-131 (1998). These techniques measure the reduction in a donor's fluorescence lifetime due to energy transfer. In a particular embodiment, a flow cytometric Foerster-type FRET technique (FCET) may be used to investigate EGFR-HER2 and HER2-HER3 dimerization, as described in Nagy et al., supra, and Brockhoff et al., *Cytometry,* 44:338-48 (2001).

FRET is preferably used in conjunction with standard immunohistochemical labeling techniques. Kenworthy, *Methods,* 24:289-96 (2001). For example, antibodies conjugated to suitable fluorescent dyes can be used as probes for labeling two different proteins. If the proteins are within proximity of one another, the fluorescent dyes act as donors and acceptors for FRET. Energy transfer is detected by standard means. Energy transfer may be detected by flow cytometric means or by digital microscopy systems, such as confocal microscopy or wide-field fluorescence microscopy coupled to a charge-coupled device (CCD) camera.

In one embodiment of the present invention, HER2 antibodies and either EGFR or HER3 antibodies are directly labeled with two different fluorophores, for example as described in Nagy et al, supra. Tumor cells or tumor cell lysates are contacted with the differentially labeled antibodies, which act as donors and acceptors for FRET in the presence of EGFR-HER2 or HER2-HER3 dimers. Alternatively, unlabeled antibodies against HER2 and either EGFR or HER3 are used along with differentially labeled secondary antibodies that serve as donors and acceptors. See, for example, Brockhoff et al., supra. Energy transfer is detected and the presence of dimers is determined if the labels are found to be in close proximity.

In other embodiments HER receptor ligands that are specific for HER2 and either EGFR or HER3 are fluorescently labeled and used for FRET studies.

In still other embodiments of the present invention, the presence of dimers on the surface of tumor cells is demonstrated by co-localization of HER2 with either EGFR or HER3 using standard direct or indirect immunofluorescence techniques and confocal laser scanning microscopy. Alternatively, laser scanning imaging (LSI) is used to detect antibody binding and co-localization of HER2 with either EGFR or HER3 in a high-throughput format, such as a microwell plate, as described in Zuck et al, *Proc. Natl. Acad. Sci. USA,* 96:11122-27 (1999).

In further embodiments, the presence of EGFR-HER2 and/or HER2-HER3 dimers is determined by identifying enzymatic activity that is dependent upon the proximity of the dimer components. A HER2 antibody is conjugated with one enzyme and an EGFR or HER3 antibody is conjugated with a second enzyme. A first substrate for the first enzyme is added and the reaction produces a second substrate for the second enzyme. This leads to a reaction with another molecule to produce a detectable compound, such as a dye. The presence of another chemical breaks down the second substrate, so that reaction with the second enzyme is prevented unless the first and second enzymes, and thus the two antibodies, are in close proximity. In a particular embodiment tumor cells or cell lysates are contacted with a HER2 antibody that is conjugated with glucose oxidase and a HER3 or EGFR antibody that is conjugated with horse radish peroxidase. Glucose is added to the reaction, along with a dye precursor, such as DAB, and catalase. The presence of dimers is determined by the development of color upon staining for DAB.

Dimers may also be detected using methods based on the eTag™ assay system (Aclara Bio Sciences, Mountain View, Calif.), as described, for example, in U.S. Patent Application 2001/0049105, published Dec. 6, 2001, both of which are expressly incorporated by reference in their entirety. An eTag™, or "electrophoretic tag," comprises a detectable reporter moiety, such as a fluorescent group. It may also comprise a "mobility modifier," which consists essentially of a moiety having a unique electrophoretic mobility. These moieties allow for separation and detection of the eTag™ from a complex mixture under defined electrophoretic conditions, such as capillary electrophoresis (CE). The portion of the eTag™ containing the reporter moiety and, optionally, the mobility modifier is linked to a first target binding moiety by a cleavable linking group to produce a first binding compound. The first target binding moiety specifically recognizes a particular first target, such as a nucleic acid or protein. The first target binding moiety is not limited in any way, and may be for example, a polynucleotide or a polypeptide. Preferably, the first target binding moiety is an antibody or antibody fragment. Alternatively, the first target binding moiety may be a HER receptor ligand or binding-competent fragment thereof.

The linking group preferably comprises a cleavable moiety, such as an enzyme substrate, or any chemical bond that may be cleaved under defined conditions. When the first target binding moiety binds to its target, the cleaving agent is introduced and/or activated, and the linking group is cleaved, thus releasing the portion of the eTag™ containing the

reporter moiety and mobility modifier. Thus, the presence of a "free" eTag™ indicates the binding of the target binding moiety to its target.

Preferably, a second binding compound comprises the cleaving agent and a second target binding moiety that specifically recognizes a second target. The second target binding moiety is also not limited in any way and may be, for example, an antibody or antibody fragment or a HER receptor ligand or binding competent ligand fragment. The cleaving agent is such that it will only cleave the linking group in the first binding compound if the first binding compound and the second binding compound are in close proximity.

In an embodiment of the present invention, a first binding compound comprises an eTag™ in which an antibody to HER2 serves as the first target binding moiety. A second binding compound comprises an antibody to EGFR or HER3 joined to a cleaving agent capable of cleaving the linking group of the eTag™. Preferably the cleaving agent must be activated in order to be able to cleave the linking group. Tumor cells or tumor cell lysates are contacted with the eTag™, which binds to HER2, and with the modified EGFR or HER3 antibody, which binds to EGFR or HER3 on the cell surface. Unbound binding compound is preferable removed, and the cleaving agent is activated, if necessary. If EGFR-HER2 or HER2-HER3 dimers are present, the cleaving agent will cleave the linking group and release the eTag™ due to the proximity of the cleaving agent to the linking group. Free eTag™ may then be detected by any method known in the art, such as capillary electrophoresis.

In one embodiment, the cleaving agent is an activatable chemical species that acts on the linking group. For example, the cleaving agent may be activated by exposing the sample to light.

In another embodiment, the eTag™ is constructed using an antibody to EGFR or HER3 as the first target binding moiety, and the second binding compound is constructed from an antibody to HER2.

In yet another embodiment, the HER dimer is detected using an antibody or other reagent which specifically or preferentially binds to the dimer as compared to binding thereof to either HER receptor in the dimer.

(ii) HER2 Phosphorylation

Phosphorylation of HER receptor may be assessed by immunoprecipitation of one or more HER receptors, such as HER2 receptor, and analysis of phosphorylated tyrosine residue(s) in the immunoprecipitated receptor(s). For example, positivity is determined by the presence of a phospho-HER2 band on the gel, using an anti-phosphotyrosine antibody to detect phosphorylated tyrosine residue(s) in the immunoprecipitated HER receptor(s). Anti-phosphotyrosine antibodies are commercially available from PanVera (Madison, Wis.), a subsidiary of Invitrogen, Chemicon International Inc. (Temecula, Calif.), or Upstate Biotechnology (Lake Placid, N.Y.). Negativity is determined by the absence of the band. Various assay formats for detecting phosphorylated proteins are contemplated including Western blot analysis, immunohistochemistry, ELISA, etc.

In one embodiment, phosphorylation of HER2 (HER2) receptor is assessed by immunohistochemistry using a phospho-specific HER2 antibody (clone PN2A; Thor et al., *J. Clin. Oncol,* 18(18):3230-3239 (2000)).

Other methods for detecting phosphorylation of HER receptor(s) include, but are not limited to, KIRA ELISA (U.S. Pat. Nos. 5,766,863; 5,891,650; 5,914,237; 6,025,145; and 6,287,784), mass spectrometry (comparing size of phosphorylated and non-phosphorylated HER2), and e-tag proximity assay with both a HER (e.g. HER2) antibody and phospho-specific or phospho-tyrosine specific antibody (e.g., using the eTagTMassay kit available from Aclara BioSciences (Mountain View, Calif.). Details of the eTag assay are described hereinabove.

One may also use phospho-specific antibodies in cellular array to detect phosphorylation status in a cellular sample of signal transduction protein (US2003/0190689).

Example 2 below describes a preferred method for determining HER2 phosphorylation by phospho-HER2 ELISA.

(iii) Gene Expression Profiling

In one embodiment, gene expression profiling can serve as a surrogate for measuring HER phosphorylation directly. This is particularly useful where the sample is a fixed sample (e.g. parrafin-embedded, formalin fixed tumor sample) where HER phosphorylation may be difficult to reliably quantify. For example, expression of two or more HER receptors and one or more HER ligand in a sample is evaluated, wherein expression of the two or more HER receptors and one or more HER ligand indicates positive HER activation in the sample. Alternatively or additionally, expression of betacellulin and/or amphiregulin in the sample can be measured, wherein betacellulin and/or amphiregulin expression indicates positive HER activation in the sample.

According to a preferred embodiment of gene expression profiling for evaluating HER2 activation, a sample from the patient is tested for expression of two or more HER receptors (preferably selected from EGFR, HER2, and HER3) and one or more HER ligands (preferably selected from betacellulin, amphiregulin, epiregulin, and TGF-α, most preferably betacellulin or amphiregulin). For example, the two or more HER receptors may be EGFR and HER2, or HER2 and HER3, and the one or more HER ligands may be betacellulin or amphiregulin. Preferably, expression of HER2 and EGFR or HER3, as well as betacellulin or amphiregulin is determined. The sample may be tested for expression of betacellulin or amphiregulin alone, or in combination with testing for expression of two or more HER receptors. Positive expression of the identified gene(s) indicates the patient is a candidate for therapy with a HER dimerization inhibitor, such as pertuzumab. Moreover, positive expression of the gene(s) indicates the patient is more likely to respond favorably to therapy with the HER dimerization inhibitor than a patient who does not have such positive expression.

Various methods for determining expression of mRNA or protein include, but are not limited to, gene expression profiling, polymerase chain reaction (PCR) including quantitative real time PCR (qRT-PCR), microarray analysis, serial analysis of gene expression (SAGE), MassARRAY, Gene Expression Analysis by Massively Parallel Signature Sequencing (MPSS), proteomics, immunohistochemistry (IHC), etc. Preferably mRNA is quantified. Such mRNA analysis is preferably performed using the technique of polymerase chain reaction (PCR), or by microarray analysis. Where PCR is employed, a preferred form of PCR is quantitative real time PCR (qRT-PCR). In one embodiment, expression of one or more of the above noted genes is deemed positive expression if it is at the median or above, e.g. compared to other samples of the same tumor-type. The median expression level can be determined essentially contemporaneously with measuring gene expression, or may have been determined previously.

The steps of a representative protocol for profiling gene expression using fixed, paraffin-embedded tissues as the RNA source, including mRNA isolation, purification, primer extension and amplification are given in various published journal articles (for example: Godfrey et al. *J. Molec. Diagnostics* 2: 84-91 (2000); Specht et al., *Am. J. Pathol.* 158:

419-29 (2001)). Briefly, a representative process starts with cutting about 10 microgram thick sections of paraffin-embedded tumor tissue samples. The RNA is then extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be included, if necessary, and RNA is reverse transcribed using gene specific promoters followed by PCR. Finally, the data are analyzed to identify the best treatment option(s) available to the patient on the basis of the characteristic gene expression pattern identified in the tumor sample examined.

Example 3 herein describes preferred methods for determining HER2 activation by gene expression profiling.

(iv) HER Expression and Amplification

To determine HER expression or amplification in the cancer, various diagnostic/prognostic assays are available. In one embodiment, HER overexpression may be analyzed by IHC, e.g. using the HERCEPTEST® (Dako). Parrafin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a HER2 protein staining intensity criteria as follows:

Score 0 no staining is observed or membrane staining is observed in less than 10% of tumor cells,

Score 1+ a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.

Score 2+ a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score 3+ a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

Those tumors with 0 or 1+ scores for HER2 overexpression assessment may be characterized as not overexpressing HER2, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing HER2.

Tumors overexpressing HER2 may be rated by immunohistochemical scores corresponding to the number of copies of HER2 molecules expressed per cell, and can been determined biochemically:

0=0-10,000 copies/cell,

1+=at least about 200,000 copies/cell,

2+=at least about 500,000 copies/cell,

3+=at least about 2,000,000 copies/cell.

Overexpression of HER2 at the 3+ level, which leads to ligand-independent activation of the tyrosine kinase (Hudziak et al., *Proc. Natl. Acad. Sci. USA,* 84:7159-7163 (1987)), occurs in approximately 30% of breast cancers, and in these patients, relapse-free survival and overall survival are diminished (Slamon et al., *Science,* 244:707-712 (1989); Slamon et al., *Science,* 235:177-182 (1987)).

Alternatively, or additionally, FISH assays such as the INFORM™ (sold by Ventana, Ariz.) or PATHVISION™ (Vysis, Ill.) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of HER2 amplification in the tumor.

In one embodiment, the cancer will be one which expresses (and may overexpress) EGFR, such expression may be evaluated as for the methods for evaluating HER2 expression as noted above.

HER receptor or HER ligand overexpression or amplification may also be evaluated using an in vivo diagnostic assay, e.g. by administering a molecule (such as an antibody) which binds the molecule to be detected and is tagged with a detectable label (e.g. a radioactive isotope) and externally scanning the patient for localization of the label.

IV. Pharmaceutical Formulations

Therapeutic formulations of the HER dimerization inhibitors used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), generally in the form of lyophilized formulations or aqueous solutions. Antibody crystals are also contemplated (see U.S. Pat Appln 2002/0136719). Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Lyophilized antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The preferred pertuzumab formulation for therapeutic use comprises 30 mg/mL pertuzumab in 20 mM histidine acetate, 120 mM sucrose, 0.02% polysorbate 20, at pH 6.0. An alternate pertuzumab formulation comprises 25 mg/mL pertuzumab, 10 mM histidine-HCl buffer, 240 mM sucrose, 0.02% polysorbate 20, pH 6.0.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Various drugs which can be combined with the HER dimerization inhibitor are described in the Method Section below. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, macroemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

V. Treatment with HER Dimerization Inhibitors

The invention herein provides a method for extending TTP or survival in a cancer patient, whose cancer displays HER activation, comprising administering a HER dimerization inhibitor to the patient in an amount which extends the patient's TTP or survival. Preferably, the HER dimerization inhibitor is a HER2 dimerization inhibitor and/or inhibits HER heterodimerization.

In one embodiment, the patient's cancer displays HER2 activation, including HER2 phosphorylation. Preferably, HER2 phosphorylation is evaluated using a phospho-ELISA assay. Alternatively, HER2 activation can be evaluated by gene expression profiling or by detecting HER dimers or heterodimers.

Examples of various cancers that can be treated with a HER dimerization inhibitor are listed in the definition section above. Preferred cancer indications include ovarian cancer; peritoneal cancer; fallopian tube cancer; breast cancer, including metastatic breast cancer (MBC); lung cancer, including non-small cell lung cancer (NSCLC); prostate cancer; and colorectal cancer. In one embodiment, the cancer which is treated is advanced, refractory, recurrent, chemotherapy-resistant, and/or platinum-resistant cancer.

Therapy with the HER dimerization inhibitor extends TTP and/or survival. In one embodiment, therapy with the HER dimerization inhibitor extends TTP or survival at least about 20% more than TTP or survival achieved by administering an approved anti-tumor agent, or standard of care, for the cancer being treated.

In the preferred embodiment, the invention provides a method for extending time to disease progression (TTP) or survival in a patient with ovarian, peritoneal, or fallopian tube cancer, whose cancer displays HER2 activation, comprising administering pertuzumab to the patient in an amount which extends the patient's TTP or survival. The patient may have advanced, refractory, recurrent, chemotherapy-resistant, and/or platinum-resistant ovarian, peritoneal or fallopian tube cancer. Administration of pertuzumab to the patient may, for example, extend TTP or survival at least about 20% more than TTP or survival achieved by administering topotecan or liposomal doxorubicin to such a patient.

The HER dimerization inhibitor is administered to a human patient in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

For the prevention or treatment of cancer, the dose of HER dimerization inhibitor will depend on the type of cancer to be treated, as defined above, the severity and course of the cancer, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician.

In one embodiment, a fixed dose of HER dimerization inhibitor is administered. The fixed dose may suitably be administered to the patient at one time or over a series of treatments. Where a fixed dose is administered, preferably it is in the range from about 20 mg to about 2000 mg of the HER dimerization inhibitor. For example, the fixed dose may be approximately 420 mg, approximately 525 mg, approximately 840 mg, or approximately 1050 mg of the HER dimerization inhibitor, such as pertuzumab.

Where a series of doses are administered, these may, for example, be administered approximately every week, approximately every 2 weeks, approximately every 3 weeks, or approximately every 4 weeks, but preferably approximately every 3 weeks. The fixed doses may, for example, continue to be administered until disease progression, adverse event, or other time as determined by the physician. For example, from about two, three, or four, up to about 17 or more fixed doses may be administered.

In one embodiment, one or more loading dose(s) of the antibody are administered, followed by one or more maintenance dose(s) of the antibody. In another embodiment, a plurality of the same dose are administered to the patient.

According to one preferred embodiment of the invention, a fixed dose of HER dimerization inhibitor (e.g. pertuzumab) of approximately 840 mg (loading dose) is administered, followed by one or more doses of approximately 420 mg (maintenance dose(s)) of the antibody. The maintenance doses are preferably administered about every 3 weeks, for a total of at least two doses, up to 17 or more doses.

According to another preferred embodiment of the invention, one or more fixed dose(s) of approximately 1050 mg of the HER dimerization inhibitor (e.g. pertzumab) are administered, for example every 3 weeks. According to this embodiment, one, two or more of the fixed doses are administered, e.g. for up to one year (17 cycles), and longer as desired.

In another embodiment, a fixed dose of approximately 1050 mg of the HER dimerization inhibitor (e.g. pertuzumab) is administered as a loading dose, followed by one or more maintenance dose(s) of approximately 525 mg. About one, two or more maintenance doses may be administered to the patient every 3 weeks according to this embodiment.

While the HER dimerization inhibitor may be administered as a single anti-tumor agent, the patient is optionally treated with a combination of the HER dimerization inhibitor, and one or more chemotherapeutic agent(s). Preferably at least one of the chemotherapeutic agents is an antimetabolite chemotherapeutic agent such as gemcitabine. The combined administration includes coadministration or concurrent administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Thus, the antimetabolite chemotherapeutic agent may be administered prior to, or following, administration of the HER dimerization inhibitor. In this embodiment, the timing between at least one administration of the antimetabolite chemotherapeutic agent and at least one administration of the HER dimerization inhibitor is preferably approximately 1 month or less, and most preferably approximately 2 weeks or less. Alternatively, the antimetabolite chemotherapeutic agent and the HER dimerization inhibitor are administered concurrently to the patient, in a single formulation or separate formulations. Treatment with the combination of the chemotherapeutic agent (e.g. antimetabolite chemotherapeutic agent such as gemcitabine) and the HER dimerization inhibitor (e.g. pertuzumab) may result in a synergistic, or greater than additive, therapeutic benefit to the patient.

An antimetabolite chemotherapeutic agent, if administered, is usually administered at dosages known therefor, or optionally lowered due to combined action of the drugs or negative side effects attributable to administration of the antimetabolite chemotherapeutic agent. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined

empirically by the skilled practitioner. Where the antimetabolite chemotherapeutic agent is gemcitabine, preferably, it is administered at a dose between about 600 mg/m^2 to 1250 mg/m^2 (for example approximately 1000 mg/m^2), for instance, on days 1 and 8 of a 3-week cycle.

Aside from the HER dimerization inhibitor and antimetabolite chemotherapeutic agent, other therapeutic regimens may be combined therewith. For example, a second (third, fourth, etc) chemotherapeutic agent(s) may be administered, wherein the second chemotherapeutic agent is either another, different antimetabolite chemotherapeutic agent, or a chemotherapeutic agent that is not an antimetabolite. For example, the second chemotherapeutic agent may be a taxane (such as paclitaxel or docetaxel), capecitabine, or platinum-based chemotherapeutic agent (such as carboplatin, cisplatin, or oxaliplatin), anthracycline (such as doxorubicin, including, liposomal doxorubicin), topotecan, pemetrexed, vinca alkaloid (such as vinorelbine), and TLK 286. "Cocktails" of different chemotherapeutic agents may be administered.

Other therapeutic agents that may be combined with the HER dimerization inhibitor include any one or more of: a second, different HER dimerization inhibitor (for example, a growth inhibitory HER2 antibody such as trastuzumab, or a HER2 antibody which induces apoptosis of a HER2-overexpressing cell, such as 7C2, 7F3 or humanized variants thereof); an antibody directed against a different tumor associated antigen, such as EGFR, HER3, HER4; anti-hormonal compound, e.g., an anti-estrogen compound such as tamoxifen, or an aromatase inhibitor; a cardioprotectant (to prevent or reduce any myocardial dysfunction associated with the therapy); a cytokine; an EGFR-targeted drug (such as TARCEVA®, IRESSA® or cetuximab); an anti-angiogenic agent (especially bevacizumab sold by Genentech under the trademark AVASTIN™); a tyrosine kinase inhibitor; a COX inhibitor (for instance a COX-1 or COX-2 inhibitor); non-steroidal anti-inflammatory drug, celecoxib (CELEBREX®); farnesyl transferase inhibitor (for example, Tipifarnib/ZARNESTRA® R115777 available from Johnson and Johnson or Lonafarnib SCH66336 available from Schering-Plough); antibody that binds oncofetal protein CA 125 such as Oregovomab (MoAb B43.13); HER2 vaccine (such as HER2 AutoVac vaccine from Pharmexia, or APC8024 protein vaccine from Dendreon, or HER2 peptide vaccine from GSK/Corixa); another HER targeting therapy (e.g. trastuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, C11033, GW572016, IMC-11F8, TAK165, etc); Raf and/or ras inhibitor (see, for example, WO 2003/86467); doxorubicin HCl liposome injection (DOXIL®); topoisomerase I inhibitor such as topotecan; taxane; HER2 and EGFR dual tyrosine kinase inhibitor such as lapatinib/GW572016; TLK286 (TELCYTA®); EMD-7200; a medicament that treats nausea such as a serotonin antagonist, steroid, or benzodiazepine; a medicament that prevents or treats skin rash or standard acne therapies, including topical or oral antibiotic; a medicament that treats or prevents diarrhea; a body temperature-reducing medicament such as acetaminophen, diphenhydramine, or meperidine; hematopoietic growth factor, etc.

Suitable dosages for any of the above coadministered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and HER dimerization inhibitor.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

Where the inhibitor is an antibody, preferably the administered antibody is a naked antibody. However, the inhibitor administered may be conjugated with a cytotoxic agent. Preferably, the conjugated inhibitor and/or antigen to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the conjugate in killing the cancer cell to which it binds. In a preferred embodiment, the cytotoxic agent targets or interferes with nucleic acid in the cancer cell. Examples of such cytotoxic agents include maytansinoids, calicheamicins, ribonucleases and DNA endonucleases.

The present application contemplates administration of the HER dimerization inhibitor by gene therapy. See, for example, WO96/07321 published Mar. 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells; in vivo and ex vivo. For in vivo delivery the nucleic acid is injected directly into the patient, usually at the site where the antibody is required. For ex vivo treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, e.g. U.S. Pat. Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for ex vivo delivery of the gene is a retrovirus.

The currently preferred in vivo nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., *J. Biol. Chem.* 262:4429-4432 (1987); and Wagner et al., *Proc. Natl. Acad. Sci. USA* 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson et al., *Science* 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

VI. Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209, USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all citations in the specification are expressly incorporated herein by reference.

Example 1

Clinical Activity of Pertuzumab in Advanced, Refractory or Recurrent Ovarian Cancer and the Role of HER2 Activation Status

This example concerns a single arm, open label, multicenter phase II clinical trial of ovarian cancer patients. Patients with advanced, refractory or recurrent ovarian cancer were treated with pertuzumab, a humanized HER2 antibody. Pertuzumab represents a new class of targeted agents called HER dimerization inhibitors (HDIs) that inhibit dimerization of HER2 with EGFR, HER3 and HER4, and inhibit signaling through MAP and P13 kinase.

65 patients with relapsed ovarian cancer were enrolled with 61 receiving therapy with "low dose" single agent pertuzumab; pertuzumab was administered intravenously (IV) with a loading of 840 mg followed by 420 mg every 3 weeks.

A second cohort of patients was treated with "high dose" pertuzumab; 1050 mg every 3 weeks, administered as a single agent. In this cohort, 64 subjects were enrolled, with 62 subjects being treated.

Tumor assessments were obtained after 2, 4, 6, 8, 12 and 16 cycles.

Response Rate (RR) by RECIST was the primary endpoint. Fresh tumor biopsies were mandatory in order to assay for HER2 phosphorylation (pHER2) status using a pHER2 enzyme-linked immunosorbent assay as described in Example 2 below. pHER2 for cohort 1 subjects was assessed. Safety and tolerability were additionally evaluated.

Secondary endpoints were TTP, duration of response, duration of survival, pharmacokinetics (PK), and FOSI (cohort 2).

Results

Baseline demographics of the patients are provided in FIG. **9**. Median age was 57 years (range 35-83) and median ECOG PS was 2. The median number of prior chemotherapy regimens was 5.

FIGS. **10-14** depict any adverse events in the treated patients. Pertuzumab was well tolerated. Diarrhea (grade 1-3) was experienced by 61% of patients. 5% of patients had a drop in ejection fraction to less than 50%.

Efficacy results are summarized in FIG. **15**. 4% of patients had a partial response (PR). 39% of patients had stable disease (SD). As shown in FIG. **16**, median TTP for patients treated with 420 mg pertuzumab was 7 weeks, and, for patients treated with 1050 mg pertuzumab was 6.6 weeks. FIG. **17** provides overall survival for patients treated with low dose or high dose pertuzumab. Median survival was 40 weeks. CA-125 responses are provided in FIG. **18**. Pertuzumab was efficacious in reducing CA-125 levels. Such reduction is an indication of therapeutic effectiveness in ovarian cancer.

HER2 activation status of patients in cohort 1, treated with 420 mg of pertuzumab, was evaluated. The results are shown in FIGS. **19-23**. Approximately 30% of ovarian cancer subjects were pHER2 positive (greater than 30% of tumor, ELISA performed as described in Example 2). Of the subjects evaluable for efficacy and pHER2 data, 26% were pHER2 positive. See FIG. **19**.

The median TTP for pHER2+ patients was 21 weeks, compared to 6 weeks in pHER2-patients, and 9 weeks in patients with unknown pHER2 status (FIGS. **20** and **22**).

Fourteen of 61 patients in cohort 1 showed evidence of pertuzumab activity. The only patient with a partial response (PR) was phospho-HER2 positive. See FIG. **21**.

Overall survival of patients was also evaluated. As shown in FIG. **23**, overall survival of pHER2 positive patients treated with pertuzumab appears superior to survival achieved with topotecan (median survival 43 weeks) or liposomal doxorubicin (36 weeks).

Conclusions

As a single agent, pertuzumab is well tolerated. Pertuzumab toxicity and efficacy do not appear to be dose-related. Pertuzumab has activity in advanced, refractory or recurrent ovarian cancer. Subjects with positive pHER2 status displayed enhanced TTP and survival efficacy compared to subjects with negative pHER2 status. Efficacy, as measured by TTP or survival, of pertuzumab in patients displaying HER2 activation appeared superior to that achieved using topotecan or liposomal doxorubicin, agents presently used to treat patients with advanced, refractory or recurrent ovarian cancer.

Example 2

PHOSPHO-HER2 Elisa for Determining HER2 Activation

Example 1 above describes the clinical trial which evaluated the efficacy of pertuzumab in subjects with advanced, refractory or recurrent ovarian cancer. This example describes development of the assay used to determine HER2 activation in the patients treated in Example 1.

The phospho-HER2 ELISA was developed to measure the concentration of HER2-associated tyrosine phosphorylation (HER2/pTyr) in human ovarian tumor tissue lysates. The assay utilizes COSTAR™ 96-well, half-area, microtiter plates because of limited sample volume. The coat antibody is an affinity purified goat anti-HER2 ECD and the secondary antibody is a biotinylated murine monoclonal (clone 4G10) specific for phosphotyrosine. The reference standard is a SK-BR-3 cell lysate with an assay range of T32 U/mL. One unit equals the amount of phosphorylated tyrosine measured in a SK-BR-3 cell lysate containing 277 pg total HER2 as determined by the Total HER2 ELISA (Total HER2 ELISA). The ELISA uses AMDEX™ streptavidin-HRP for detection and TMB as the substrate.

Materials

1. Standard Material, SK-BR-3 Cell lysate 1,056 U/mL HER2/pTyr
2. Control Source, SK-BR-3 Cell lysate 1,056 U/mL
3. Coat antibody, goat anti-HER2 ECD 9.6 mg/mL
4. Secondary antibody, biotinylated murine anti-phosphotyrosine, clone 4G10, 971 µg/mL (Upstate Biotech Cat #16-103)
5. AMDEX™ Streptavidin conjugated to HRP (SA-HRP) (Amersham Biosciences Catalog No. RPN4401)
6. Substrate, Tetramethyl Benzidine (TMB) Peroxidase Substrate (Kirkegaard & Perry Labs [KPL] Catalog No. 50-76-01)
7. Coat Buffer, 0.05 M sodium carbonate buffer, pH 9.6

8. Assay Diluent, PBS/0.5% BSA/0.05% Polysorbate 20/0.05% PROCLIN 300™, pH 7.4
9. Lysis Buffer: Base Lysis Buffer (50 mM Tris-HCl/150 mM NaCl/5 mM EDTA/1% TRITON X-100™)/1:10 Protease Inhibitor Cocktail/1:100 Phosphatase Inhibitor Cocktail I/1:100 Phosphatase Inhibitor Cocktail II/50 mM sodium fluoride/2 mM sodium ortho-vanadate, pH 8.1
10. Sample, Standard, and Control Diluent: Lysis Buffer
11. MDA-468 (ATCC# HTB-132). HER2 Expression level: None. Tissue: human mammary gland, breast, adenocarcinoma
12. MCF-7 (ATCC# HTB-22). HER2 Expression level: 0 (normal expression levels of HER2). Tissue: human mammary gland; breast; epithelial; metastatic site: pleural effusion adenocarcinoma
13. SK-BR-3 (ATCC# HTB-30, Manassas, Va.). HER2 Expression level: 3 (high level HER2 overexpression). Tissue: human mammary gland; breast; metastatic site: pleural effusion adenocarcinoma
14. BT-474 (ATCC# HTB-20, Manassas, Va.). HER2 Expression level: 3 (high level HER2 overexpression) Tissue: human mammary gland; breast; duct; ductal carcinoma
15. BT-474 Tumor Lysates. Mice were inoculated with BT-474. After 2 weeks tumors were harvested. Harvest tumors were homogenized to produce tumor lysates

Preparation of Materials

Standard Material/Stock: The phospho HER2 ELISA Standard Stock is neat Standard Material. The Standard Material was prepared by collecting lysates from three 245× 245 mm cell culture trays containing SK-BR-3 (SKBR3) cells, which were 80% to 90% confluent. Cell lysates clarified by centrifugation and the supernatant was collected. The supernatant is used as the Standard Material.

The Standard Material was assigned a concentration of 1,056 U/mL so that the lowest calibrator in the assay reporting range would be 1 U/mL. One unit is defined as the amount of phosphorylated tyrosine measured in a SK-BR-3 cell lysate containing 277 pg total HER2 as determined by the Total HER2

Cell lysate controls: Cell lysate controls were prepared from the Standard Material. Standard Material was diluted in Lysis Buffer to obtain HER2/pTyr levels that represent the low, middle, and high ranges of the assay standard curve.

Tissue lysate controls: Tissue lysate controls were prepared from the BT474 tumor lysates. BT474 tumor lysates were diluted in Lysis Buffer to obtain HER2/pTyr levels that represent the high range of the assay standard curve.

Coat source: Goat anti-HER2 ECD Stock I was prepared by diluting the source material (9.6 mg/mL) to 100 g/mL in PBS.

Biotinylated conjugate: The biotinylated murine anti-phosphotyrosine antibody (1 μg/mL) was purchased from Upstate Biotech. The antibody is a biotinylated, protein A-purified, monoclonal IgG2b-kappa raised against phosphotyramine coupled to KLH. The biotinylated monoclonal antiphosphotyrosine antibody (clone 4G10, Cat #05-321) is specific for phosphotyrosine and does not cross-react with phosphoserine or phosphothreonine.

Specificity of goat anti-HER2 ECD: HER2 receptor activation initiates when receptor dimerization occurs with other family members. Unless signaling is strictly due to HER2 homodimerization, EGFR, HER3, and/or HER4 must be expressed within the active tumor. Each of these receptors may be present in ovarian tissue lysate samples and could interfere in accurately measuring HER2-associated tyrosine phosphorylation (HER2/pTyr) if these receptors cross-react to the coat antibody.

The specificity of the goat anti-HER2 ECD antibody was determined by surface plasmon resonance analysis (BIACORE 3000®, BIACORE® International AB, Neuchatel, Switzerland). The goat anti-HER2 ECD antibody was immobilized onto a CM5 sensor chip using amine coupling chemistry. The sensor chip was blocked with 1 M ethanolamine-HCl, pH 8.5, and conditioned with 10 mM HCl. Specificity was determined by injecting soluble recombinant EGFR (sEGFR) (Research Diagnostics, Inc., Flanders, N.J.) and recombinant human HER2 ECD/human IgG1 Fc fusion proteins over the immobilized goat anti-HER2 antibody. The fusion proteins consisted of the ECD of HER2, HER3, or HER4 fused to the carboxy-terminal 6× histidine-tagged Fc region of human IgG1 via a peptide linker (R&D Systems, Minneapolis, Minn.).

Reference subtracted relative responses for sEGFR, HER3-Fc, and HER4-Fc were –41 RU, 0.3 RU, and 2.5 RU, respectively. The negative relative response obtained for sEGFR was due to refractive index changes between the mobile phase (HBS-EP) and the sample excipient. The relative response for HER2-Fc was 374 RU.

Methods

The phospho HER2 ELISA utilizes COSTAR™ half-area (A/2) plates coated with goat anti-HER2 ECD at 4 μg/mL in 0.5 M sodium carbonate buffer, pH 9.6, and incubated 18-72 hours at 2° C.-8° C. The wells are blocked with approximately 150 μL/well assay diluent for 1-2 hours and then 50 μL/well of standards, controls, and samples are added. The minimum dilution for ovarian tumor tissue lysates is 1/40 in Lysis Buffer. The standards, controls, and samples are incubated 2 hours at ambient temperature with agitation. The wells are washed with PBS/0.05 TWEEN 20™ and 250 ng/mL of biotinylated anti-phosphotyrosine is added. After 2 hours, the wells are washed and AMDEX™ streptavidin-HRP is added. AMDEX™ streptavidin-HRP (SA-HRP) is a polymeric conjugate with multiple enzyme labels linked to the streptavidin. After 15 minutes the wells are washed and a tetramethylbenzidine substrate (TMB) is added and allowed to develop for 15 minutes before being stopped with 1 M phosphoric acid. The absorbance is measured using a SPECTRAMAX™ plate reader (Molecular Devices Corp., Sunnyvale, Calif.) with a 450 nm filter and a 650 nm reference filter. The sample concentrations are calculated relative to a nonlinear, four-parameter logistic fit of a seven-point standard curve (Marquardt, D. *J. Soc. Indust. Appl. Math.* 431-441 (1963)). The assay range of the ELISA is 1 to 32 U/mL. The units are arbitrary units, where 1 U equals the amount of phosphorylated tyrosine measured in a SK-BR-3 cell lysate containing 277 pg total HER2. The lower limit of the assay was set to 1 U/mL and was defined by the lower limit of detection.

Precision of the phospho HER2 ELISA was re-evaluated after the Standard Material concentration was re-assigned. Intra- and inter-assay precision were evaluated by determining the coefficient of variation (CV) of HER2/pTyr in a SKBR3 cell lysate at three different levels. The SKBR3 cell lysate was diluted to obtain HER2 levels that represent the low, middle, and high ranges of the assay standard curve. After the Standard Material concentration was re-assigned the High Control was not within the high range of the assay standard curve. Therefore, a BT474 tissue lysate control was diluted to fall within the high range. The lysates, which were run as assay controls, were analyzed in duplicate over 5 days.

The control data were imported into STATVIEW for ANOVA™ analysis to determine the intra- and inter-assay standard deviation.

The CVs were calculated as follows:

100×(Standard Deviation)/(mean control value)

The intra-assay precision CVs were 4%, 4%, 3%, and 11% for the BT474, High, Mid, and Low controls, respectively. The inter-assay precision CVs were 5%, 6%, 5%, and 14% for the BT474, High, Mid, and Low controls, respectively.

During the development of the phospho HER2 ELISA, a SKBR3 cell lysate was diluted to 22.9, 6.39, and 1.71 U/mL in neat MDA468 cell lysate. The samples were diluted in Lysis buffer containing SKBR3 HER2/pTyr to maintain a constant level of HER2/pTyr throughout the entire dilution series while matrix effects are diluted out. The dilution series was analyzed in the Phospho HER2 ELISA and compared to SKBR3 HER2/pTyr without MDA468 to determine recovery. Percent recovery was calculated as follows:

100×SKBR3 HER2/pTyr diluted in MDA468/SKBR3 HER2/pTyr diluted in Lysis Buffer

The results for SKBR3 HER2/pTyr recovery at the three levels in the presence of MDA468 cell lysate, revealed the matrix significantly enhances recovery in sample dilutions between neat and 1/16 at the 1.71 U/mL level, with HER2/pTyr recoveries between 120% and 127%. Matrix interference was not observed at any other level. Recovery between 80% and 120% is demonstrated in each level starting at a sample dilution of 1/16 in Lysis Buffer.

Ovarian and BT474 tumor lysates were serially diluted two-fold in Lysis Buffer and analyzed in the Phospho HER2 ELISA. The starting dilution for the BT474 samples, which were analyzed during development, was 1/20. The ovarian lysates were analyzed after the Standard Material concentration was re-assigned. The starting dilution for the ovarian lysates varied according to expected HER2/pTyr concentrations.

The percent difference for the dilution series, which is an indicator of sample dilution linearity, was calculated as follows:

$$100 \times \frac{\left(\begin{array}{c}\text{greatest Corrected Result value} - \\ \text{lowest Corrected Result value}\end{array}\right)}{\left(\begin{array}{c}\text{average of greatest and} \\ \text{lowest Corrected Result values}\end{array}\right)}$$

Percent differences for ovarian tissue lysate HF8198 were calculated starting at a dilution of either 1/80, 1/160, or 1/320. Percent differences for ovarian tissue lysate HF7945 were calculated starting at a dilution of either 1/320, 1/640, or 1/1280. Percent differences for the remaining ovarian tissue lysates were calculated starting at a dilution of either 1/20, or 1/40, to determine the minimum dilution as well as assess the linearity of dilution.

The differences for the BT474 dilution series ranged from −9% to 12%. The differences for HF7930 and HF7934 dilution series starting at a 1/10 dilution were 43% and 38%, respectively. The differences for HF8197 were 2%, 8%, and 5% for dilution series starting at 1/320, 1/160, and 1/1280, respectively. The differences for HF8198 were 72%, 34%, and 3%, for dilution series starting at 1/80, 1/160, and 1/320, respectively.

Eighteen different ovarian tissue lysates were analyzed in the phospho HER2 ELISA after the Standard Material concentration was changed. Samples were diluted two-fold starting from 1/20 to 1/160. One sample was LTR at a 1/20 dilution; 10 samples were LTR at a 1/40 dilution. Seven samples had measurable levels of HER2/pTyr at 1/20 and 1/40 dilutions and one sample had measurable levels of HER2/pTyr up to a 1/80 dilution. The differences for samples that had measurable levels of HER2/pTyr at 1/20 and 1/40 dilutions ranged from 16% to 34%, with three of seven samples with differences less than 20%. The one sample that had measurable levels of HER2/pTyr up to a 1/80 dilution, sample HF7931, had differences of 16% and 4% for dilution series starting at 1/20 and 1/40, respectively.

Pertuzimab and trastuzumab were analyzed in the phospho HER2 ELISA to determine if these therapeutics interfere. The antibodies were diluted to concentrations ranging from 1.5 to 10,000 ng/mL in heregulin stimulated MCF7 (MCF7+) cell lysates containing 98.48 U/mL HER2/pTyr.

During development, cell and tissue lysates were subjected to four cycles of freezing and thawing to determine the effects of temperature cycling. Frozen SKBR3 cell lysate and BT474 tumor lysates were thawed at ambient temperature. From each lysate 10 μL were removed and diluted in Assay Diluent. The remaining lysates were flash frozen in a mixture of dry ice and methanol and thawed again. Test samples were once again removed and diluted in Assay Diluent (first freeze/thaw cycle, 1×). The flash freeze, thaw and sample collection procedure was repeated twice to obtain samples from the second and third freeze/thaw cycles (2× and 3×, respectively).

Diluted samples were assayed in the phospho HER2 ELISA to determine HER2/pTyr recovery with respect to the "fresh" sample. The "fresh" sample is the sample taken from the initial thawing.

Recovery of SKBR3 HER2/pTyr for 1×, 2×, and 3× samples were 104%, 109%, and 113%, respectively. Recovery of BT474 HER2/pTyr in sample 314A were 99%, 103%, and 99%, for 1×, 2×, and 3× samples, respectively. Recovery of BT474 HER2/pTyr in sample 365 were 111%, 96%, and 99%, for 1×, 2×, and 3× samples, respectively.

The Lower Limit of Quantitation (LLOQ) was set as the average concentration of the low control, 1.35 U/mL. Because the low control is included within each experiment, it is a reliable indicator of the lower limit to which samples can be accurately measured. Therefore, the minimum quantifiable concentration in the phospho HER2 ELISA is the LLOQ multiplied by the minimum sample dilution (1/40), or 54 U/mL.

Conclusions

A sensitive and accurate ELISA was developed to measure HER2-associated tyrosine phosphorylation (HER2/pTyr) in tumor tissue lysates. The phospho HER2 ELISA demonstrated sensitivity down to 1.35 U/mL with a minimum quantifiable concentration of 54 U/mL, where 1 U is equal to the amount of phosphorylated tyrosine measured in a SK-BR-3 cell lysate containing 277 pg total HER2. The phospho-HER2 ELISA demonstrated good precision at four levels. The intra-assay precision CVs were 4%, 3%, 3%, and 11%, for the BT474 tissue lysate control and the High, Mid, and Low SKBR3 cell lysate controls, respectively. The inter-assay precision CVs were 5%, 6%, 5%, and 14%, for the BT474 tissue lysate control and the High, Mid, and Low SKBR3 cell lysate controls, respectively.

The phospho HER2 ELISA demonstrated good recovery of HER2/pTyr in the presence MDA468 cell lysate. Starting at a 1/16 dilution, recoveries ranged from 88% to 120%. The

ELISA demonstrated high specificity as EGFR, HER3-IgG Fc, and HER4-IgG Fc do not cross-react with the assay coat.

Human ovarian tumor and BT474 mouse xenograft tumor tissue lysates were used to analyze linearity of dilution and minimum sample dilution. The differences of dilution corrected values for the BT474 tumor lysates ranged from −9% to 12%. Out of the seven ovarian lysates that had measurable levels of HER2/pTyr at 1/20 and 1/40 dilutions, only three had differences less than 20%, while six out of seven had differences less than or equal to 23%. The one sample that had measurable levels of HER2/pTyr up to a 1/80 dilution, sample HF7931, had a difference of 4% for dilution series starting at 1/40. All of the above samples did not meet the ≤20% criteria at a minimum dilution of 1/20, therefore, the minimum sample dilution will be 1/40.

The BT474 tissue lysates and human ovarian tissue lysate samples HF8197 and HF8198 had high measurable levels of HER2/pTyr and required dilutions between 1/80 to 1/320 to fall within the quantitative range of the assay. The BT474 samples and sample HF8197, which had the highest measured HER2/pTyr concentration within the human ovarian tumor tissue subset, diluted linearly throughout the entire assay range. In contrast, sample HF8198 diluted nonlinearly as the corrected for dilution HER2/pTyr concentrations monotonically increased throughout the assay range and appear to plateau at a 1/320 dilution.

SKBR3 cell lysates subjected to three freeze/thaw cycles demonstrated very good recovery. HER2/pTyr recovery ranged from 104% to 113% with respect to the same freshly thawed sample.

Two BT474 tumor lysates were also subjected to three freeze/thaw cycles. BT474 phospho HER2 recovery ranged from 99% to 103%. HER2/pTyr recovery from BT474 ranged from 96% to 111%. Therefore, temperature cycling does not appear to effect phospho HER2 activity.

The phospho HER2 ELISA does not demonstrate any interference from either pertuzimab or trastuzumab.

Example 3

Gene Expression Profiling for Determining HER2 Activation

This example shows how HER2 activation can be evaluated by determining gene expression profiles as an alternative to determining HER2 phosphorylation directly. This profiling may be done on fresh, frozen, or formalin-fixed, paraffin-embedded ovarian tumor specimens, but preferably the latter.

Ovarian cancer specimens treated with pertuzumab were profiled for gene expression using AFFYMETRIX® microarray analysis performed according to the manufacturer's instructions. The microarray expression data was analyzed to identify gene patterns which would be associated with HER2 phosphorylation status. Remarkably, a pattern emerged where tumors with relatively high levels of expression of EGFR, HER2, HER3, and the HER ligand betacelullin were also positive for HER2 phosphorylation. The correlation was positive in six of the six HER2 phosphorylation positive cases, and none of the HER2 phosphorylation negative cases were predicted positive using microarray expression data as the basis for the algorithm.

In a second analysis, prediction of HER2 phosphorylation status was achieved by using a single gene only, namely betacellulin. All six HER2 phosphorylation positive tumors had a betacellulin expression above the median, again using microarray expression data.

A second method for quantifying gene expression, quantitative real time polymerase chain reaction (qRT-PCR), was used to validate, and was compared with, the microarray data. qRT-PCT would be a preferred method for measuring gene expression in the typical patient sample available in a clinical setting. Diagnostic technology platforms are already established for this method. qRT-PCR was performed as described in Cronin et al., *Am. J. Pathol.* 164(1):35-42 (2004); and Ma et al., *Cancer Cell* 5:607-616 (2004). RNA was extracted from frozen ovarian tumors using commercially available reagents from Qiagen, Valencia, Calif. Primers and probes for TAQMAN™ qRT-PCR analysis were designed to give amplicon lengths of about 100 bases or less. Transcripts were quantitated by qRT-PCR using a TAQMAN™ instrument (Applied BioSystems), with expression levels of the test genes normalized to those of the reference genes. The "house keeping" gene GUS was selected as the control gene because of its low variance and high expression.

Based on the experiments noted above an algorithm was developed based on gene expression profiling date of tumors with known HER2 phosphorylation status by ELISA. A tumor is deemed positive for a gene expression profile associated with HER2 phosphorylation that has betacellulin or amphiregulin and HER2 expression at the median or above and/or EGFR and/or HER3 expression at the median or above. Alternatively, expression of betacellulin or amphiregulin alone can be measured by qRT-PCR to identify tumors with predicted phosphorylation of HER2.

SEQUENCE LISTING

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<213> ORGANISM: Mus musculus

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Ile Gly Val Ala Trp Tyr Gln Gln Arg Pro Gly Gln Ser Pro Lys
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Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Asp
                50                  55                  60

Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile
                65                  70                  75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu
                95                 100                 105

Ile Lys


<210> SEQ ID NO 2
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 2

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Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Thr Phe Thr
                20                  25                  30

Asp Tyr Thr Met Asp Trp Val Lys Gln Ser His Gly Lys Ser Leu
                35                  40                  45

Glu Trp Ile Gly Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                50                  55                  60

Asn Gln Arg Phe Lys Gly Lys Ala Ser Leu Thr Val Asp Arg Ser
                65                  70                  75

Ser Arg Ile Val Tyr Met Glu Leu Arg Ser Leu Thr Phe Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
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Phe Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
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<400> SEQUENCE: 3

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                20                  25                  30

Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105
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Ile Lys

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20 25 30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
50 55 60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
95 100 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
110 115

<210> SEQ ID NO 5
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence is synthesized

<400> SEQUENCE: 5

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
20 25 30

Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys

<210> SEQ ID NO 6
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence is synthesized

<400> SEQUENCE: 6

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  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                20                  25                  30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
                95                 100                 105

Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
               110                 115


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<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
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<223> OTHER INFORMATION: Sequence is synthesized.
<220> FEATURE:
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<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

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Lys Gly


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Lys Ala Ser Gln Asp Val Ser Ile Gly Val Ala
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<223> OTHER INFORMATION: Xaa is preferably R or L
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<222> LOCATION: 6
<223> OTHER INFORMATION: Xaa is preferably Y or E
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<222> LOCATION: 7
<223> OTHER INFORMATION: Xaa is preferably T or S

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<210> SEQ ID NO 12
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 12

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<210> SEQ ID NO 13
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 13

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Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser
20 25 30

Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
110 115 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
125 130 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
140 145 150

```
Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
                200                 205                 210

Arg Gly Glu Cys


<210> SEQ ID NO 14
<211> LENGTH: 448
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 14

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Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
                20                  25                  30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
                110                 115                 120

Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
                125                 130                 135

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp
                140                 145                 150

Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu
                155                 160                 165

Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly
                170                 175                 180

Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
                185                 190                 195

Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn
                200                 205                 210

Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr
                215                 220                 225

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
                230                 235                 240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
                245                 250                 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
                260                 265                 270
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Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
                275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
                290                 295                 300

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp
                305                 310                 315

Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
                320                 325                 330

Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
                335                 340                 345

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met
                350                 355                 360

Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
                365                 370                 375

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
                380                 385                 390

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
                395                 400                 405

Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
                410                 415                 420

Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
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Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
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<210> SEQ ID NO 15
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 15

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Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                 20                  25                  30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
                110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
                125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
                140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
                155                 160                 165
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Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
                170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
                185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
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Arg Gly Glu Cys


<210> SEQ ID NO 16
<211> LENGTH: 449
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence is synthesized

<400> SEQUENCE: 16

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
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Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                95                 100                 105

Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                 120

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser
                125                 130                 135

Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys
                140                 145                 150

Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
                155                 160                 165

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
                170                 175                 180

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
                185                 190                 195

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
                200                 205                 210

Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
                215                 220                 225

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                230                 235                 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
                260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
                275                 280                 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
                290                 295                 300
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Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
                305                 310                 315

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
                320                 325                 330

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
                335                 340                 345

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
                350                 355                 360

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
                365                 370                 375

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
                380                 385                 390

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
                395                 400                 405

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
                410                 415                 420

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
                425                 430                 435

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
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<210> SEQ ID NO 17
<211> LENGTH: 217
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
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<223> OTHER INFORMATION: Sequence is synthesized.

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Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln
                20                  25                  30

Asp Val Ser Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys
                35                  40                  45

Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly
                50                  55                  60

Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
                65                  70                  75

Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr
                80                  85                  90

Cys Gln Gln Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr
                95                 100                 105

Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile
                110                 115                 120

Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val
                125                 130                 135

Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln
                140                 145                 150

Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser
                155                 160                 165

Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
                170                 175                 180

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
                185                 190                 195
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Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys
                200                 205                 210

Ser Phe Asn Arg Gly Glu Cys
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<210> SEQ ID NO 18
<211> LENGTH: 449
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence is synthesized.

<400> SEQUENCE: 18

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  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
                20                  25                  30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
                50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
                95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
                110                 115                 120

Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
                125                 130                 135

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp
                140                 145                 150

Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu
                155                 160                 165

Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly
                170                 175                 180

Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
                185                 190                 195

Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn
                200                 205                 210

Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr
                215                 220                 225

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
                230                 235                 240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
                245                 250                 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
                260                 265                 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
                275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
                290                 295                 300

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp
                305                 310                 315
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Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
                320                 325                 330

Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
                335                 340                 345

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met
                350                 355                 360

Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
                365                 370                 375

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
                380                 385                 390

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
                395                 400                 405

Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
                410                 415                 420

Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
                425                 430                 435

Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
                440                 445


<210> SEQ ID NO 19
<211> LENGTH: 195
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 19

Thr Gln Val Cys Thr Gly Thr Asp Met Lys Leu Arg Leu Pro Ala
  1               5                  10                  15

Ser Pro Glu Thr His Leu Asp Met Leu Arg His Leu Tyr Gln Gly
                 20                  25                  30

Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr Leu Pro Thr
                 35                  40                  45

Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly
                 50                  55                  60

Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln
                 65                  70                  75

Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
                 80                  85                  90

Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr
                 95                 100                 105

Pro Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu
                110                 115                 120

Arg Ser Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg
                125                 130                 135

Asn Pro Gln Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile
                140                 145                 150

Phe His Lys Asn Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn
                155                 160                 165

Arg Ser Arg Ala Cys His Pro Cys Ser Pro Met Cys Lys Gly Ser
                170                 175                 180

Arg Cys Trp Gly Glu Ser Ser Glu Asp Cys Gln Ser Leu Thr Arg
                185                 190                 195


<210> SEQ ID NO 20
<211> LENGTH: 124
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
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<400> SEQUENCE: 20

Thr Val Cys Ala Gly Gly Cys Ala Arg Cys Lys Gly Pro Leu Pro
1 5 10 15

Thr Asp Cys Cys His Glu Gln Cys Ala Ala Gly Cys Thr Gly Pro
20 25 30

Lys His Ser Asp Cys Leu Ala Cys Leu His Phe Asn His Ser Gly
35 40 45

Ile Cys Glu Leu His Cys Pro Ala Leu Val Thr Tyr Asn Thr Asp
50 55 60

Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg Tyr Thr Phe Gly
65 70 75

Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu Ser Thr Asp
80 85 90

Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln Glu Val
95 100 105

Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys Pro
110 115 120

Cys Ala Arg Val

<210> SEQ ID NO 21
<211> LENGTH: 169
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 21

Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu Val Arg Ala Val
1 5 10 15

Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys Lys Ile Phe
20 25 30

Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp Pro Ala
35 40 45

Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe Glu
50 55 60

Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
65 70 75

Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile
80 85 90

Arg Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln
95 100 105

Gly Leu Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu
110 115 120

Gly Ser Gly Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe
125 130 135

Val His Thr Val Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln
140 145 150

Ala Leu Leu His Thr Ala Asn Arg Pro Glu Asp Glu Cys Val Gly
155 160 165

Glu Gly Leu Ala

<210> SEQ ID NO 22
<211> LENGTH: 142
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 22

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Cys His Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro
  1               5                  10                  15

Thr Gln Cys Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys
                20                  25                  30

Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val
                35                  40                  45

Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
                50                  55                  60

Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val
                65                  70                  75

Ala Cys Ala His Tyr Lys Asp Pro Pro Phe Cys Val Ala Arg Cys
                80                  85                  90

Pro Ser Gly Val Lys Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys
                95                 100                 105

Phe Pro Asp Glu Glu Gly Ala Cys Gln Pro Cys Pro Ile Asn Cys
               110                 115                 120

Thr His Ser Cys Val Asp Leu Asp Asp Lys Gly Cys Pro Ala Glu
               125                 130                 135

Gln Arg Ala Ser Pro Leu Thr
               140
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What is claimed is:

1. A method for extending time to disease progression (TTP) or survival in cancer patients with metastatic breast cancer which displays HER activation comprising: (a) administering a HER2 antibody which comprises the variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4 as fixed doses of 420 mg to the patients and (b) measuring TTP or survival in the patients to confirm it is extended.

2. The method of claim **1** wherein the HER2 antibody inhibits HER heterodimerization.

3. The method of claim **1** wherein the cancer displays HER2 activation.

4. The method of claim **3** wherein the cancer displays HER2 overexpression or amplification.

5. The method of claim **1** wherein the HER2 antibody is pertuzumab.

6. The method of claim **1** wherein the HER2 antibody is a naked antibody.

7. The method of claim **1** wherein the HER2 antibody is an intact antibody.

8. The method of claim **1** wherein the HER2 antibody is an antibody fragment comprising an antigen binding region.

9. The method of claim **1** wherein the HER2 antibody is administered as a single anti-tumor agent.

10. The method of claim **1** comprising administering a second therapeutic agent to the patients.

11. The method claim **10** wherein the second therapeutic agent is selected from the group consisting of chemotherapeutic agent, HER antibody, antibody directed against a tumor associated antigen, anti-hormonal compound, cardioprotectant, cytokine, EGFR-targeted drug, anti-angiogenic agent, tyrosine kinase inhibitor, COX inhibitor, non-steroidal anti-inflammatory drug, farnesyl transferase inhibitor, antibody that binds oncofetal protein CA 125, HER2 vaccine, HER targeting therapy, Raf or ras inhibitor, liposomal doxorubicin, topotecan, taxane, dual tyrosine kinase inhibitor, TLK286, EMD-7200, a medicament that treats nausea, a medicament that prevents or treats skin rash or standard acne therapy, a medicament that treats or prevents diarrhea, a body temperature-reducing medicament, and a hematopoietic growth factor.

12. The method of claim **10** wherein the second therapeutic agent comprises trastuzumab.

13. The method of claim **1** wherein TTP is extended.

14. The method of claim **1** wherein survival is extended.

15. The method of claim **1** wherein administration of the HER2 antibody extends TTP or survival at least about 20% more than TTP or survival achieved by administering an approved anti-tumor agent to the cancer patients.

16. A method for extending time to disease progression (TTP) or survival in breast cancer patients comprising: (a) administering a HER2 antibody to the patients as fixed doses of about 420 mg of the HER2 antibody so as to extend TTP or survival in the patients, wherein the HER2 antibody comprises the variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4, respectively, and (b) measuring TTP or survival in the patients to confirm it is extended.

17. The method of claim **16** which extends TTP.

18. The method of claim **16** which extends survival.

19. The method of claim **16** wherein the patients are metastatic breast cancer patients.

20. The method of claim **19** wherein the cancer displays HER2 activation.

21. The method of claim **20** wherein the cancer displays amplified or overexpressed HER2.

22. The method of claim **16** wherein the fixed doses of about 420 mg of the HER2 antibody are administered about every three weeks.

23. The method of claim **22** wherein a loading dose of 840 mg of the HER2 antibody is administered followed by the fixed doses of about 420 mg of the HER2 antibody.

24. The method of claim **16** comprising administering a second therapeutic agent to the patients.

25. The method of claim **24** wherein the second therapeutic agent comprises trastuzumab.

26. The method of claim **16** wherein the HER2 antibody is pertuzumab.

27. A method for extending time to disease progression (TTP) or survival in breast cancer patients comprising: (a) administering pertuzumab to the patients as a loading dose of 840 mg followed by fixed doses of about 420 mg of the pertuzumab every three weeks and (b) measuring TTP or survival to confirm it is extended in the patients.

28. The method of claim **27** which extends TTP.

29. The method of claim **27** which extends survival.

30. The method of claim **27** wherein the patients have metastatic breast cancer.

31. The method of claim **27** wherein the cancer displays HER2 activation.

32. The method of claim **31** wherein the cancer displays amplified or overexpressed HER2.

33. The method of claim **27** comprising administering a second therapeutic agent to the patients.

34. The method of claim **33** wherein the second therapeutic agent comprises trastuzumab.

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