```
        10         20         30         40         50         60
GATCCCGAGGTTATGCTGGTTGAATCTGGTGGAGTACTGATGGAACCTGGTGGGTCCCTG
 D  P  E  V  M  L  V  E  S  G  G  V  L  M  E  P  G  G  S  L
                                  ScaI                EcoO

        70         80         90        100        110        120
AAGCTGAGCTGTGCTGCTAGCGGCTTCACGTTCTCTCGTTACGCCATGTCTTGGGTCCGT
 K  L  S  C  A  A  S  G  F  T  F  S  R  Y  A  M  S  W  V  R
   EspI        NheI                          PflMI

       130        140        150        160        170        180
CAGACTCCGGAGAAGCGTCTAGAGTGGGTCGCGACGATATCTTCTGGTGGTTCGAACACT
 Q  T  P  E  K  R  L  E  W  V  A  T  I  S  S  G  G  S  N  T
      BspMII      XbaI       NruI   EcoRV          AsuII

       190        200        210        220        230        240
TACTATCCAGACAGTGTGAAGGGTCGATTCACGATCTCTCGAGACAACGCTAAGAACACG
 Y  Y  P  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  T
                                  XhoI

       250        260        270        280        290        300
TTGTACCTGCAAATGTCTTCTCTACGTAGTGAAGATACTGCTATGTACTACTGTGCACGT
 L  Y  L  Q  M  S  S  L  R  S  E  D  T  A  M  Y  Y  C  A  R
    BspMI+           SnaBI                          ApaLI

       310        320        330        340        350        360
CCTCCACTGATCTCACTAGTTGCTGATTATGCCATGGATTATTGGGGTCATGGTGCTAGC
 P  P  L  I  S  L  V  A  D  Y  A  M  D  Y  W  G  H  G  A  S
               SpeI             NcoI                    NheI

       370        380        390        400        410        420
GTTACTGTGAGCTCTGGTGGCGGTGGGTCGGGCGGTGGTGGCTCGGGTGGCGGCGGATCG
 V  T  V  S  S  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S
       SacI

       430        440        450        460        470        480
GATATCGTTATGACTCAGTCTCATAAGTTCATGTCCACTTCTGTTGGTGACCGTGTTTCT
 D  I  V  M  T  Q  S  H  K  F  M  S  T  S  V  G  D  R  V  S
EcoRV                                        BstEII

       490        500        510        520        530        540
ATCACTTGTAAGGCCAGCCAGGATGTGGGTGCTGCTATCGCATGGTATCAGCAGAAGCCC
 I  T  C  K  A  S  Q  D  V  G  A  A  I  A  W  Y  Q  Q  K  P
               PflMI                                    Sma

       550        560        570        580        590        600
GGGCAGTCTCCTAAGCTGCTGATCTACTGGGCGTCGACTCGTCATACTGGTGTCCCGGAT
 G  Q  S  P  K  L  L  I  Y  W  A  S  T  R  H  T  G  V  P  D
I                              SalI

       610        620        630        640        650        660
CGTTTCACTGGGTCCGGATCAGGTACTGATTTCACTCTGACTATTTCGAACGTTCAGTCT
 R  F  T  G  S  G  S  G  T  D  F  T  L  T  I  S  N  V  Q  S
          BspMII                                AsuII

       670        680        690        700        710        720
GATGACCTGGCTGATTACTTCTGCCAGCAATATTCCGGGTACCCTCTGACTTTCGGTGCC
 D  D  L  A  D  Y  F  C  Q  Q  Y  S  G  Y  P  L  T  F  G  A
                           SspI    KpnI                Nae

       730        740        750
GGCACTAAACTCGAGCTGAAGTAACTGCAG
 G  T  K  L  E  L  K  *
I       XhoI          PstI
```

FIG. 9E

```
                                        10                                  20
Met Lys Ala Ile Phe Val Leu Lys Gly Ser Leu Asp Arg Asp Leu Asp Ser Arg Leu Asp
ATG AAA GCA ATT TTC GTA CTG AAA GGT TCA CTG GAC AGA GAT CTG GAC TCT CGT CTG GAT
                                                 BglII
                                        30                                  40
Leu Asp Val Arg Thr Asp His Lys Asp Leu Ser Asp His Leu Val Leu Val Asp Leu Ala
CTG GAC GTT CGT ACC GAC CAC AAA GAC CTG TCT GAT CAC CTG GTT CTG GTC GAC CTG GCT
                                           BclI                  SalI
                                        50                                  60
Arg Asn Asp Leu Ala Arg Ile Val Thr Pro Gly Ser Arg Tyr Val Ala Asp Leu Glu Phe
CGT AAC GAC CTG GCT CGT ATC GTT ACT CCC GGG TCT CGT TAC GTT GCG GAT CTG GAA TTC
                                        SmaI                                EcoRI

Asp
GAT
```

FIG. 10A

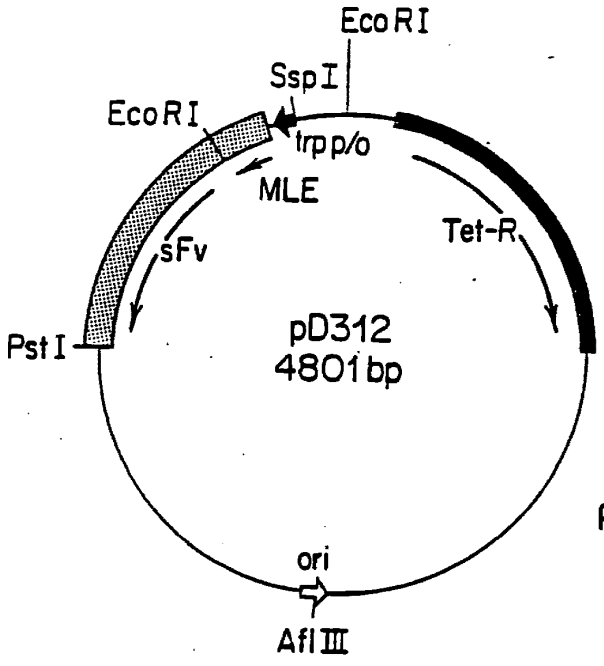

FIG. 10B

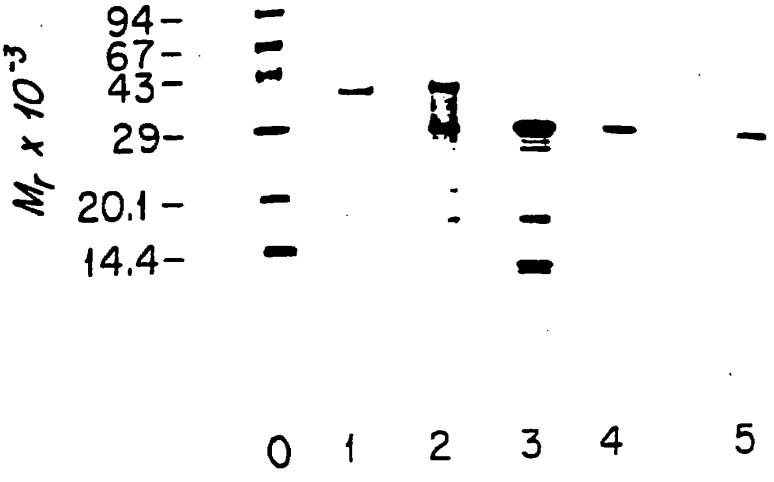

FIG. 11

```
D V Q L Q E S G P G L V K P S Q S L S L T C S V T G Y S I T
S G Y F W N W I R Q F P G N K L E W L G F I K Y D G S N Y G
N P S L K N R V S I T R D T S E N Q F F L K L D S V T T A T
Y Y C A G D N D H L Y F D Y W G Q G T T L T V S

G G G G S G G G G S G G G G S

Q A V V T Q E S A L T T S P G G T V I L T C R S S T G A V T
T S N Y A N W I Q E K P D H L F T G L I G G T S N R A P G V
P V R F S G S L I G D K A A L T I T G A Q T E D D A M Y F C
A L W F R N H F V F G G G T K V T V L G
```

FIG. 9C

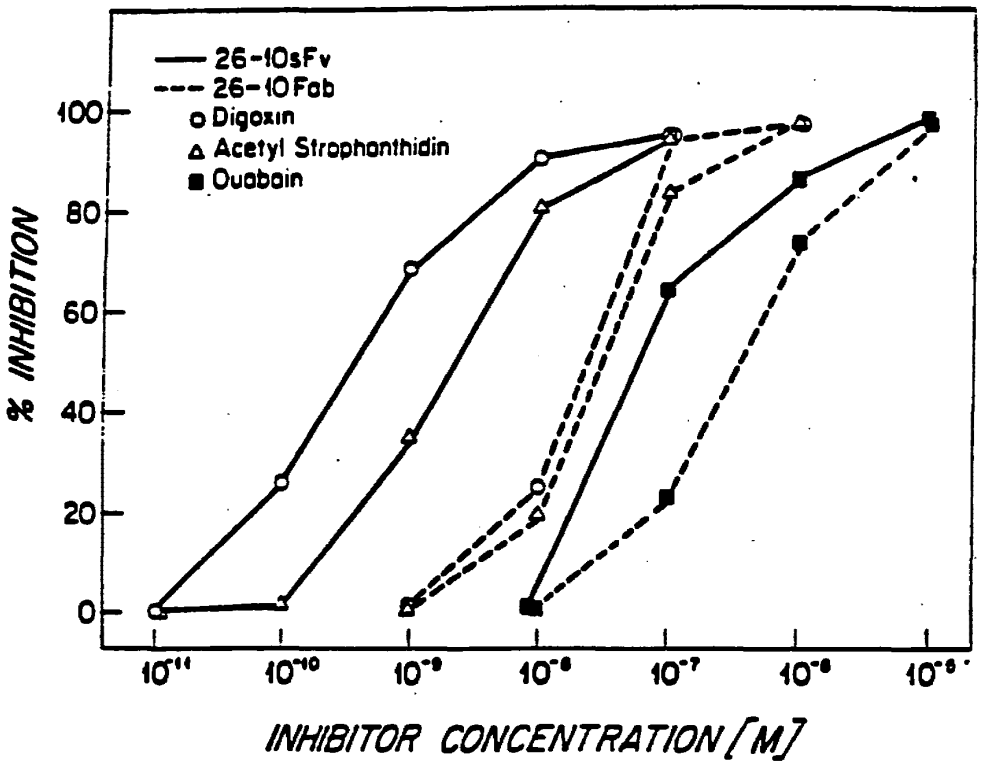
26-10sFv
26-10Fab
Digoxin
Acetyl Strophanthidin
Ouabain
% INHIBITION
100
80
60
40
20
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
INHIBITOR CONCENTRATION [M]

FIG. 12

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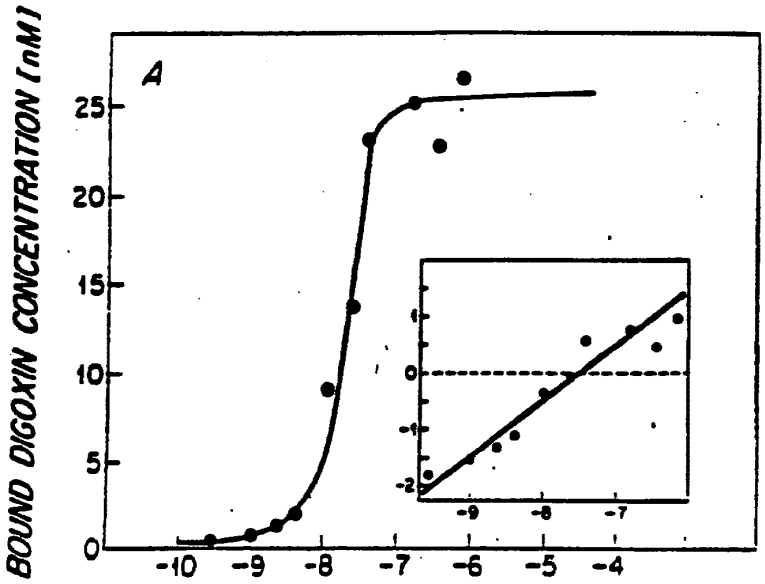

FIG. 13A

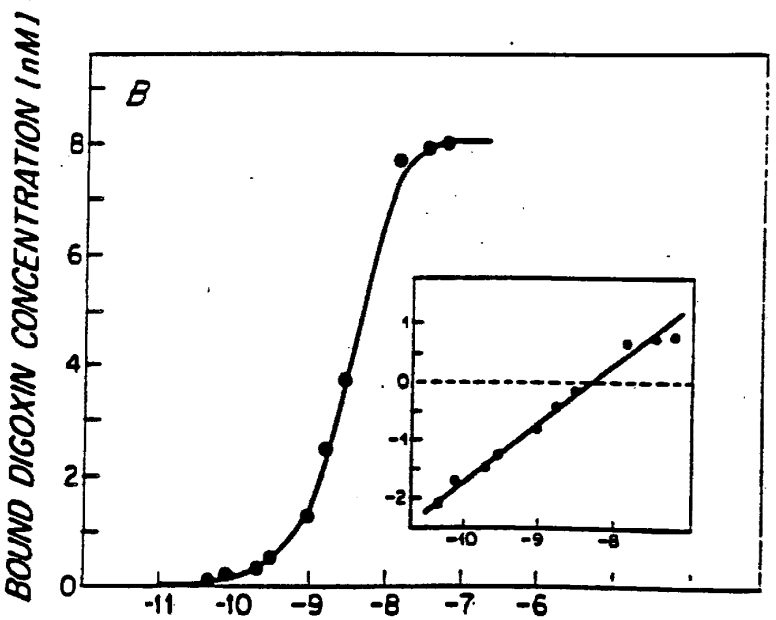

FIG. 13B

```
        10         20         30         40         50         60
GAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCGTTCTACGAGATCTTG
 E  F  M  A  D  N  K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L
EcoRI                                      MluI       BglII
                                           XmnI

        70         80         90        100        110        120
CACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAG
 H  L  P  N  L  N  E  E  Q  R  N  G  F  I  Q  S  L  K  D  E
 BspMI+                                      HindIII

       130        140        150        160        170        180
CCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAACGATGCGCAGGCACCG
 P  S  Q  S  A  N  L  L  A  D  A  K  K  L  N  D  A  Q  A  P
                  NheI                          FspI

       190        200        210        220        230        240
AAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCG
 K  S  D  Q  G  Q  F  M  A  D  N  K  F  N  K  E  Q  Q  N  A
                                                    MluI
                                                   XmnI

       250        260        270        280        290        300
TTCTACGAGATCTTGCACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAA
 F  Y  E  I  L  H  L  P  N  L  N  E  E  Q  R  N  G  F  I  Q
       BglII     BspMI+                                      H

       310        320        330        340        350        360
AGCTTGAAGGATGAGCCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAAC
 S  L  K  D  E  P  S  Q  S  A  N  L  L  A  D  A  K  K  L  N
indIII                              NheI

       370        380
GATGCGCAGGCACCGAAATCGGATCC
 D  A  Q  A  P  K  S  D  P
  FspI              BamHI
```

FIG. 14

(BABS)-

```
        10         20         30         40         50         60         70
GGATCCGGTAACTCTGACTCTGAATGCCCGCTGAGCCACGACGGGTACTGCCTGCACGACGGTGTTTGCATGTAC
 G  S  G  N  S  D  S  E  C  P  L  S  H  D  G  Y  C  L  H  D  G  V  C  M  Y
BamHI                 BsmI+   EspI

        85         95        105        115        125        135        145
ATCGAAGCTCTGGACAAATACGCATGCAACTGCGTTGTAGGCTACATCGGTGAGCGCTGCCAGTATCGCGATCTG
 I  E  A  L  D  K  Y  A  C  N  C  V  V  G  Y  I  G  E  R  C  Q  Y  R  D  L
                    SphI                                                NruI

       160        170
AAATGGTGGGAGCTGCGTTAACTGCAG
 K  W  W  E  L  R  *
              HpaI PstI
```

FIG. 15A

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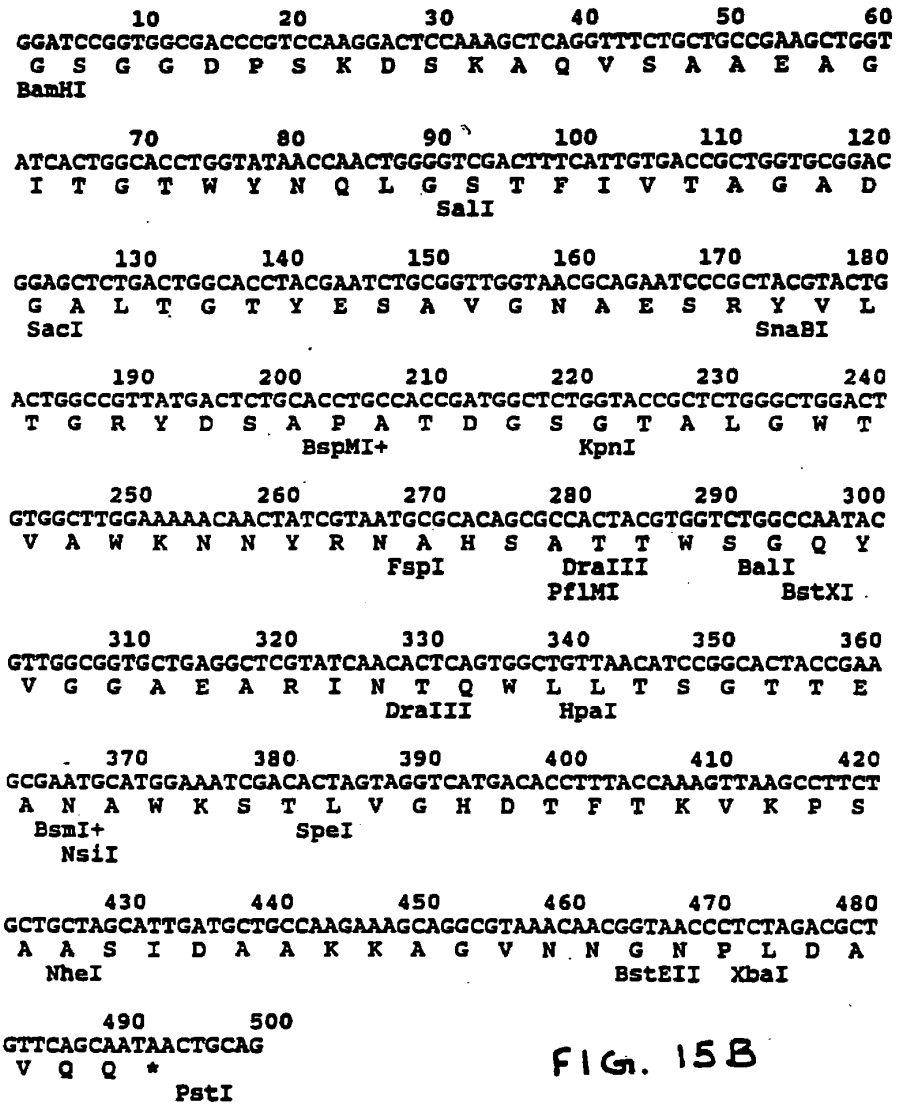
(BABS)-
10 20 30 40 50 60
GGATCCGGTGGCGACCCGTCCAAGGACTCCAAAGCTCAGGTTTCTGCTGCCGAAGCTGGT
G S G G D P S K D S K A Q V S A A E A G
BamHI
70 80 90 100 110 120
ATCACTGGCACCTGGTATAACCAACTGGGGTCGACTTTCATTGTGACCGCTGGTGCGGAC
I T G T W Y N Q L G S T F I V T A G A D
SalI
130 140 150 160 170 180
GGAGCTCTGACTGGCACCTACGAATCTGCGGTTGGTAACGCAGAATCCCGCTACGTACTG
G A L T G T Y E S A V G N A E S R Y V L
SacI
SnaBI
190 200 210 220 230 240
ACTGGCCGTTATGACTCTGCACCTGCCACCGATGGCTCTGGTACCGCTCTGGGCTGGACT
T G R Y D S A P A T D G S G T A L G W T
BspMI+
KpnI
250 260 270 280 290 300
GTGGCTTGGAAAAACAACTATCGTAATGCGCACAGCGCCACTACGTGGTCTGGCCAATAC
V A W K N N Y R N A H S A T T W S G Q Y
FspI
DraIII
BalI
PflMI
BstXI
310 320 330 340 350 360
GTTGGCGGTGCTGAGGCTCGTATCAACACTCAGTGGCTGTTAACATCCGGCACTACCGAA
V G G A E A R I N T Q W L L T S G T T E
DraIII
HpaI
370 380 390 400 410 420
GCGAATGCATGGAAATCGACACTAGTAGGTCATGACACCTTTACCAAAGTTAAGCCTTCT
A N A W K S T L V G H D T F T K V K P S
BsmI+
SpeI
NsiI
430 440 450 460 470 480
GCTGCTAGCATTGATGCTGCCAAGAAAGCAGGCGTAAACAACGGTAACCCTCTAGACGCT
A A S I D A A K K A G V N N G N P L D A
NheI
BstEII
XbaI
490 500
GTTCAGCAATAACTGCAG
V Q Q *
PstI

FIG. 15B

(BABS)- 26/31

```
        10         20         30         40         50         60
GGATCCGGTGTACGTAGCTCCTCTCGCACTCCGTCCGATAAGCCGGTTGCTCATGTAGTT
 G  S  G  V  R  S  S  S  R  T  P  S  D  K  P  V  A  H  V  V
BamHI      SnaBI

        70         80         90        100        110        120
GCTAACCCTCAGGCAGAAGGTCAGCTTCAGTGGCTGAACCGTCGCGCTAACGCCCTGCTG
 A  N  P  Q  A  E  G  Q  L  Q  W  L  N  R  R  A  N  A  L  L
      MstII                                                BglI

       130        140        150        160        170        180
GCAAACGGCGTTGAGCTCCGTGATAACCAGCTCGTGGTACCTTCTGAAGGTCTGTACCTG
 A  N  G  V  E  L  R  D  N  Q  L  V  V  P  S  E  G  L  Y  L
            SacI          PflMI     KpnI

       190        200        210        220        230        240
ATCTATTCTCAAGTACTGTTCAAGGGTCAGGGCTGCCCGTCGACTCATGTTCTGCTGACT
 I  Y  S  Q  V  L  F  K  G  Q  G  C  P  S  T  H  V  L  L  T
          ScaI                       SalI

       250        260        270        280        290        300
CACACCATCAGCCGTATTGCTGTATCTTACCAGACCAAAGTTAACCTGCTGAGCGCTATC
 H  T  I  S  R  I  A  V  S  Y  Q  T  K  V  N  L  L  S  A  I
                                    HpaIBspMI+  Eco47III
                                          EspI

       310        320        330        340        350        360
AAGTCTCCGTGCCAGCGTGAAACTCCCGAGGGTGCAGAAGCGAAACCATGGTATGAACCG
 K  S  P  C  Q  R  E  T  P  E  G  A  E  A  K  P  W  Y  E  P
                                          NcoI

       370        380        390        400        410        420
ATCTACCTGGGTGGCGTATTTCAACTGGAGAAAGGTGACCGTCTGTCCGCAGAAATCAAC
 I  Y  L  G  G  V  F  Q  L  E  K  G  D  R  L  S  A  E  I  N
                                BstEII

       430        440        450        460        470        480
CGTCCTGACTATCTAGATTTCGCTGAATCTGGCCAGGTGTACTTCGGTATTATCGCACTG
 R  P  D  Y  L  D  F  A  E  S  G  Q  V  Y  F  G  I  I  A  L
          XbaI                  BalI

       490
TAACTGCAG
*
  PstI
```

FIG. 15C

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(BABS) -

```
       10         20         30         40         50         60
GGATCCGGTGCTGATCAGCTGACTGACGAGCAGATCGCTGAATTTAAAGAGGCTTTCTCT
 G  S  G  A  D  Q  L  T  D  E  Q  I  A  E  F  K  E  A  F  S
BamHI      BclIPvuII                        DraI

       70         80         90         100        110        120
CTGTTTGACAAAGACGGTGACGGTACCATCACTACCAAAGAGCTCGGCACCGTTATGCGC
 L  F  D  K  D  G  D  G  T  I  T  T  K  E  L  G  T  V  M  R
                   KpnI                SacI              FspI

       130        140        150        160        170        180
AGCCTTGGCCAGAACCCGACTGAAGCTGAATTGCAGGACATGATCAACGAAGTCGACGCT
 S  L  G  Q  N  P  T  E  A  E  L  Q  D  M  I  N  E  V  D  A
    BalI                                 BclI       SalI

       190        200        210        220        230        240
GACGGTAACGGCACCATCGATTTTCCGGAATTTCTGAACCTGATGGCGCGCAAGATGAAA
 D  G  N  G  T  I  D  F  P  E  F  L  N  L  M  A  R  K  M  K
             ClaI    BspMII                BssHII

       250        260        270        280        290        300
GACACTGACTCTGAAGAGGAACTGAAAGAGGCCTTCCGTGTTTTCGACAAAGACGGTAAC
 D  T  D  S  E  E  E  L  K  E  A  F  R  V  F  D  K  D  G  N
                         StuI

       310        320        330        340        350        360
GGTTTCATCTCGGCCGCTGAACTGCGTCACGTTATGACTAACCTGGGTGAAAAGCTTACT
 G  F  I  S  A  A  E  L  R  H  V  M  T  N  L  G  E  K  L  T
       EagI                                          HindIII

       370        380        390        400        410        420
GACGAAGAAGTTGACGAAATGATTCGCGAAGCTGACGTCGATGGTGACGGCCAGGTTAAC
 D  E  E  V  D  E  M  I  R  E  A  D  V  D  G  D  G  Q  V  N
             XmnI    NruI      AatII                HpaI

       430        440        450
TACGAAGAGTTCGTTCAGGTTATGATGGCTAAGTAACTGCAG
 Y  E  E  F  V  Q  V  M  M  A  K  *
                                  PstI
```

FIG. 15D

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(BABS)-

```
        10         20         30         40         50         60
GGATCCGGTGGAGGCTCTCTGGGCTCTCTGACTATTGCCGAACCGGCAATGATTGCTGAA
 G  S  G  G  G  S  L  G  S  L  T  I  A  E  P  A  M  I  A  E
BamHI                                  BglI                Bsm

        70         80         90        100        110        120
TGCAAGACTCGTACCGAAGTCTTCGAGATCTCTCGTCGTCTGATCGATCGCACTAATGCC
 C  K  T  R  T  E  V  F  E  I  S  R  R  L  I  D  R  T  N  A
I+                        BglII            ClaI              Bs
                                            PvuI

       130        140        150        160        170        180
AACTTCCTGGTATGGCCGCCGTGCGTCGAGGTACAACGCTGCTCCGGGTGTTGCAACAAT
 N  F  L  V  W  P  P  C  V  E  V  Q  R  C  S  G  C  C  N  N
tXI

       190        200        210        220        230        240
CGTAACGTTCAATGTCGACCGACTCAAGTCCAGCTGCGTCCGGTCCAAGTCCGCAAAATC
 R  N  V  Q  C  R  P  T  Q  V  Q  L  R  P  V  Q  V  R  K  I
             SalI               PvuII

       250        260        270        280        290        300
GAGATTGTACGTAAGAAACCGATCTTTAAGAAGGCCACTGTTACTCTGGAAGACCATCTG
 E  I  V  R  K  K  P  I  F  K  K  A  T  V  T  L  E  D  H  L
       SnaBI

       310        320        330        340        350
GCATGCAAATGTGAGACTGTAGCGGCCGCACGTCCAGTTACTTAACTGCAG
 A  C  K  C  E  T  V  A  A  A  R  P  V  T  *
SphI                  EagI                    PstI
                      NotI
```

FIG. 15E

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(BABS)-

```
        10         20         30         40         50         60
GGATCCGGTATATTCCCCAAACAATACCCAATTATAAACTTTACCACAGCGGGTGCCACT
 G  S  G  I  F  P  K  Q  Y  P  I  I  N  F  T  T  A  G  A  T
BamHI

        70         80         90        100        110        120
GTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCGGTCGTTTAACAACTGGAGCTGAT
 V  Q  S  Y  T  N  F  I  R  A  V  R  G  R  L  T  T  G  A  D

       130        140        150        160        170        180
GTGAGACATGAAATACCAGTGTTGCCAAACAGAGTTGGTTTGCCTATAAACCAACGGTTT
 V  R  H  E  I  P  V  L  P  N  R  V  G  L  P  I  N  Q  R  F

       190        200        210        220        230        240
ATTTTAGTTGAACTCTCAAATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACC
 I  L  V  E  L  S  N  H  A  E  L  S  V  T  L  A  L  D  V  T
                                                Eco47III

       250        260        270        280        290        300
AATGCATATGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACAAT
 N  A  Y  V  V  G  Y  R  A  G  N  S  A  Y  F  F  H  P  D  N
    NdeI
NsiI

       310        320        330        340        350        360
CAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAATCGATATACATTC
 Q  E  D  A  E  A  I  T  H  L  F  T  D  V  Q  N  R  Y  T  F
                                                ClaI

       370        380        390        400        410        420
GCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGCTGGTAATCTGAGAGAAAATATC
 A  F  G  G  N  Y  D  R  L  E  Q  L  A  G  N  L  R  E  N  I

       430        440        450        460        470        480
GAGTTGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTTATTATTACAGTACTGGT
 E  L  G  N  G  P  L  E  E  A  I  S  A  L  Y  Y  Y  S  T  G
                                    Eco47III        ScaI

       490        500        510        520        530        540
GGCACTCAGCTTCCAACTCTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAA
 G  T  Q  L  P  T  L  A  R  S  F  I  I  C  I  Q  M  I  S  E

       550        560        570        580        590        600
GCAGCAAGATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGAGA
 A  A  R  F  Q  Y  I  E  G  E  M  R  T  R  I  R  Y  N  R  R
                                 FspI                    Bgl
```

FIG. 15F

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(BABS) -

```
        10         20         30         40         50         60
GGATCCGGTGCTCCGACTTCTAGCTCTACTAAGAAAACTCAGCTTCAGCTGGAACACCTG
 G  S  G  A  P  T  S  S  S  T  K  K  T  Q  L  Q  L  E  H  L
BamHI                                              PvuII

        70         80         90        100        110        120
CTGCTGGACCTTCAGATGATCCTGAACGGTATCAACAACTACAAGAACCCGAAACTGACT
 L  L  D  L  Q  M  I  L  N  G  I  N  N  Y  K  N  P  K  L  T

       130        140        150        160        170        180
CGTATGCTGACTTTCAAATTCTACATGCCGAAGAAAGCTACCGAACTGAAACACCTTCAG
 R  M  L  T  F  K  F  Y  M  P  K  K  A  T  E  L  K  H  L  Q

       190        200        210        220        230        240
TGCCTGGAAGAAGAACTGAAGCCGCTGGAGGAAGTACTGAACCTGGCTCAGTCTAAAAAC
 C  L  E  E  E  L  K  P  L  E  E  V  L  N  L  A  Q  S  K  N
                                ScaI

       250        260        270        280        290        300
TTCCACCTGCGTCCGCGTGACCTGATCAGCAACATCAACGTAATCGTTCTAGAACTTAAA
 F  H  L  R  P  R  D  L  I  S  N  I  N  V  I  V  L  E  L  K
                   BclI                          XbaI

       310        320        330        340        350        360
GGCTCTGAAACTACCTTCATGTGCGAATACGCTGACGAAACTGCTACCATCGTAGAATTT
 G  S  E  T  T  F  M  C  E  Y  A  D  E  T  A  T  I  V  E  F

       370        380        390        400        410        420
CTGAACCGTTGGATCACCTTCTGCCAGTCTATCATCTCTACTCTGACTTAACTGCAG
 L  N  R  W  I  T  F  C  Q  S  I  I  S  T  L  T  *
                                                  PstI
```

FIG. 15G

(BABS) -

```
        10         20         30         40         50         60
GGATCCGGTGCTGACAACAAATTCAACAAGGAACAGCAGAACGCGTTCTACGAGATCTTG
 G  S  G  A  D  N  K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L
BamHI                                    MluI       BglII
                                         XmnI

        70         80         90        100        110        120
CACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAG
 H  L  P  N  L  N  E  E  Q  R  N  G  F  I  Q  S  L  K  D  E
 BspMI+                                    HindIII

       130        140        150        160        170        180
CCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAACGATGCGCAGGCACCG
 P  S  Q  S  A  N  L  L  A  D  A  K  K  L  N  D  A  Q  A  P
                  NheI                          FspI

       190        200        210        220        230        240
AAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCG
 K  S  D  Q  G  Q  F  M  A  D  N  K  F  N  K  E  Q  Q  N  A
                                                        MluI
                                                       XmnI

       250        260        270        280        290        300
TTCTACGAGATCTTGCACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAA
 F  Y  E  I  L  H  L  P  N  L  N  E  E  Q  R  N  G  F  I  Q
       BglII    BspMI+                                      H

       310        320        330        340        350        360
AGCTTGAAGGATGAGCCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAAC
 S  L  K  D  E  P  S  Q  S  A  N  L  L  A  D  A  K  K  L  N
indIII                              NheI

       370        380
GATGCGCAGGCACCGAAATAACTGCAG
 D  A  Q  A  P  K  *
  FspI          PstI
```

FIG. 15H

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01737

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 13/00, C12P 21/00, C12N 15/00, C07H 15/12

U.S. CL.: 530/287, 435/68, 435/172.3, 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/387,388 935/6,9,10,11,15,22,23,24,59,60 435/68,170,172.3, 240.1 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstract Data Base (CAS) 1967-1988; BIOSIS DATA BASE 1969-1988 Keywords: antigen, binding, site, synthetic, biosynthesis, See attachment.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	U.S., A, 4,642,334 (MOORE ET AL) 10 February 1987. See abstract and columns 2, 3, 25 and 26.	1-48
Y	BIOCHEMISTRY, Volume 17 issued 1978, September (Washington, D.C., U.S.A.), (M.S. ROSEMBLATT ET AL.) "Isolation of an active variable-domain fragment from a homogeneous rabbit antibody heavy chain, physiochemical and immunological properties", See pages 3877-3882, See particularly page 3877.	1-48
Y	BIOCHEMISTRY, Volume 19, issued 1980, August (Washington, D.C. U.S.A.), (P.H. EHRLICH ET AL), "Isolation of an active heavy-chain variable domain from a homogeneous rabbit antibody by cathepsin B digestion of the aminoethylated heavy chain" See pages 4091-4096. See particularly page 4091.	1-48

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 August 1988	08 SEP 1988
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Richard Peet

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Attachment to PCT/ISA/210
II. Field Searched

Keywords Continued

immunoglobulin, specificity, variable, region, domain, chimeric, heavy, light, Fv, antibody, antibodies, cancer, tumor, treatment.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES, U.S.A., Volume 81, issued 1984, November (Washington, D.C., U.S.A.), (MORRISON ET AL.), "Chimeric human antibody molecules: mouse antigen binding domains with human constant region domains", See pages 6851-6855. See particularly pages 6851 and 6855.	1-48
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES U.S.A., Volume 84, issued 1987, January (Washington, D.C., U.S.A.), (L.K. SUN ET AL "Chimeric antibody with human constant regions and mouse variable regions directed against carcinoma-associated antigen 17-A", See pages 214-218. See particularly page 214.	1-48
Y	PROCEEDINGS NATIONAL ACADEMY SCIENCES U.S.A., Volume 82, issued 1985, May (Washington, D.C., U.S.A.), (S. OHNO ET AL), "Antigen-binding specificities of antibodies are primarily determined by seven residues of V_H", See pages 2945-2949. See particularly page 2945.	1-48
Y	CHEMICAL ABSTRACTS, Volume 107, No. 5, issued 1987, August 3 (Columbus, Ohio, U.S.A.), A.P. RICHARDSON ET AL., "The radiolabeling of antibodies for use in tumor location and treatment", See page 330 column 1, the abstract no. 35697n, Med. Sci. Res. 1987, 15(7), 343-7 (Eng.)	1-48
Y	CHEMICAL ABSTRACTS, Volume 105, No. 5, issued 1986, August 4 (Columbus, Ohio, U.S.A.), R. ARNON "Immuno targeted chemotherapy in cancer research", See page 1, column 2, abstract no. 34903y. Proc. FEBS Congr. 16th 1984 (Pub. 1985). B, 565-70 (Eng.)	1-48

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	CHEMICAL ABSTRACTS, Volume 106, No. 13, issued 1987, March 30 (Columbus, Ohio U.S.A.), H.J. THIESEN ET AL "Selective Killing of human bladder cancer cells by combined treatment with A and B chain ricin antibody conjugates", See page 37, column 1, abstract no. 95759g. Cancer Res., 1987, 47(2), 419-423 (Eng.)	1-48
Y	CHEMICAL ABSTRACTS, Volume 105, No. 11, issued 1986, September 15 (Columbus, Ohio, U.S.A.), M.S. NEUBERGER "Novel antibodies by DNA transfection", see page 475, column 2, abstract no. 95609d, Adv. Immunopharmacol. 3., Proc. Int. Conf. 3rd 1985 (Pub. 1986), 317-327 (Eng.)	1-48
Y	WO, A, WO 86/0090 DAW 3 January 1986 (03.01.86) See pages 1-16, See particularly pages 2-9.	1-48
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES, U.S.A. Volume 81, issued 1984, June (Washington, D.C. U.S.A.), (S. CABILLY ET AL.) "Generation of antibody activity from immunoglobulin polypeptide chains produced in Escherichia coli" See pages 3273-3277. See particularly pages 3273 and 3275.	1-48
X Y	WO, A, WO 86/01533 DAW 13 March 1986 (13.03.86) See pages 1-32. See particularly pages 3-8.	11 1-10, 12-48
E	U.S. A, 4,704,692 (R.C. LADNER) 3 November 1987. See abstract and columns 2 and 14.	1-48

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(57) Abstract

A reshaped human antibody or reshaped human antibody fragment having specificity for human placental alkaline phosphatase (PLAP) is produced by transferring the complementarity determining regions (CDRs) from a murine anti-PLAP hybridoma cell line H17E2 into a human antibody variable region framework. The reshaped molecule can be used in the treatment or diagnosis of cancer.

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SPECIFIC BINDING AGENTS

This invention relates to specific binding agents, and in particular to polypeptides containing amino acid sequences that bind specifically to other proteinaceous or non-proteinaceous materials. The invention most particularly concerns the production of such specific binding agents by genetic engineering.

Antibody structure

Natural antibody molecules consist of two identical heavy-chain and two identical light-chain polypeptides, which are covalently linked by disulphide bonds. Figure 13 of the accompanying drawings diagramatically represents the typical structure of an antibody of the IgG class. Each of the chains is folded into several discrete domains. The N-terminal domains of all the chains are variable in sequence and therefore called the variable regions (V-regions). The V-regions of one heavy (VH) and one light chain (VL) associate to form the antigen-binding site. The module formed by the combined VH and VL domains is referred to as the Fv (variable fragment) of the

antibody. The C-terminal ends of both heavy and light chains are more conserved in sequence and chains are more conserved in sequence and therefore referred to as the constant regions. Heavy chain constant regions are composed of several domains, eg. the heavy chain of the gamma-isotype (IgG) consists of three domains (CH1, CH2, CH3) and a hinge region which connects the CH1 and CH2 domains. The hinges of the two heavy chains are covalently linked together by disulphide bridges. Light chains have one constant domain which packs against the CH1 domain. The constant regions of the antibody molecule are involved in effector functions such as complement lysis and clearing by Antibody Dependant Cell Cytotoxicity (ADCC). Classical digestion of an antibody with the protease papain yields three fragments. One fragment contains the CH2 and CH3 domains and, as it crystallises easily, was called the Fc fragment. The other two fragments were designated the Fab (antigen-binding) fragments, they are identical and contain the entire light chain combined with the VH and CH1 domain. When using pepsin, the proteolytic cleavage is such that the two Fabs remain connected via the hinge and form the $(Fab)_2$ fragment. Each of the domains is represented by a separate exon at the genetic level.

The variable regions themselves each contain 3 clusters of hypervariable residues, in a framework of more conserved sequences. These hypervariable regions interact with the antigen, and are called the Complementarity Determining Regions (CDRs). The more conserved sequences are called the Framework Regions (FRs). See Kabat et al (1987). X-ray studies of antibodies have shown that the CDRs form loops which protrude from the top of the molecule, whilst the FRs provide a structural beta-sheet framework.

Modified antibodies

In one embodiment, the invention relates to so-called "reshaped" or "altered" human antibodies, ie. immunoglobulins having essentially human constant and framework regions but in which the complementarity determining regions (CDRs) correspond to those found in a non-human immunoglobulin, and also to corresponding reshaped antibody fragments.

The general principles by which such reshaped human antibodies and fragments may be produced are now well-known, and reference can be made to Jones et al (1986), Riechmann et al (1988), Verhoeyen et al (1988), and EP-A-239400 (Winter). A comprehensive list of relevant literature references is provided later in this specification.

Reshaped human antibodies and fragments have particular utility in the in-vivo diagnosis and treatment of human ailments because the essentially human proteins are less likely to induce undesirable adverse reactions when they are administered to a human patient, and the desired specificity conferred by the CDRs can be raised in a host animal, such as a mouse, from which antibodies of selected specificity can be obtained more readily. The variable region genes can be cloned from the non-human antibody, and the CDRs grafted into a human variable-region framework by genetic engineering techniques to provide the reshaped human antibody or fragment. To achieve this desirable result, it is necessary to identify and sequence at least the CDRs in the selected non-human antibody, and preferably the whole non-human variable region sequence, to allow

identification of potentially important CDR-framework interactions.

Summary of the invention

The invention provides, as one embodiment, a synthetic specific binding polypeptide having specificity for human placental alkaline phosphatase (PLAP). By synthetic, we particularly mean that the polypeptide is produced by recombinant DNA technology, and to that extent at least is different from a naturally-occurring or naturally-induced specific binding agent having identical specificity. Alternatively, the synthetic polypeptide has been produced by artificially assembling a sequence of amino acids to produce a novel or nature-identical molecule. The synthetic polypeptide can be equivalent to an intact conventional antibody, or equivalent to a multiple or single-chain fragment of such an antibody, or can be simply a material that includes one or more sequences that confer the desired specific binding capability.

The invention provides as an important embodiment, a reshaped human antibody, or a reshaped human antibody fragment, having anti-human placental alkaline phosphatase (PLAP) specificity.

More particularly, the invention provides a reshaped human antibody or reshaped human antibody fragment, having anti-human placental alkaline phosphatase specificity, containing one or more of the CDRs depicted in Figures 1 and 2 of the accompanying drawings. Preferably, the reshaped antibody or fragment of the invention contains all 3 of the CDRs depicted in Figure 1 of the accompanying drawings, in a human heavy chain variable region

framework. Alternatively, or in addition, the reshaped antibody or fragment of the invention contains all 3 of the CDRs depicted in Figure 2 of the accompanying drawings, in a human light chain variable region framework.

Another embodiment of the invention is a reshaped antibody or reshaped antibody fragment containing a protein sequence as depicted in Figure 10 and/or Figure 11 of the accompanying drawings.

Other important embodiments of the invention are an expression vector incorporating a DNA sequence as depicted in Figure 10 and/or Figure 11 of the accompanying drawings, and an expression vector incorporating a DNA sequence encoding one or more of the protein sequences designated as being a CDR in Figure 1 and/or Figure 2 of the accompanying drawings.

An important aspect of the invention is a stable host cell line containing a foreign gene that causes the host cell line to produce a specific binding agent according to the invention. This can be a stable host cell line containing a foreign gene that encodes at least one of the amino acid sequences designated as being a CDR in Figure 1 and/or Figure 2 of the accompanying drawings, together with a protein framework that enables the encoded amino acid sequence when expressed to function as a CDR having specificity for PLAP.

The invention particularly provides an immortalised mammalian cell line, or a yeast, or other eukaryotic cell, or a prokaryotic cell such as a bacterium, producing a reshaped antibody or fragment according to the invention.

Another important aspect of the invention is a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, having specificity equivalent to that of the gamma-1, kappa anti-PLAP monoclonal antibody secreted by murine hybridoma cell line H17E2.

The invention also provides two novel plasmids, pSVgptHu2VHPLAP-HuIgG1 and pSVneoHuVkPLAP-HuCk, and these plasmids can be used in the production of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

These plasmids are contained in novel E.coli strains NCTC 12389 and NCTC 12390, respectively.

Other aspects of the invention are:

a) A DNA sequence encoding a reshaped human antibody heavy-chain variable region having specificity for human placental alkaline phosphatase, as contained in E.coli NCTC 12389.

b) A DNA sequence encoding a reshaped human antibody light-chain variable region having specificity for human placental alkaline phosphatase, as contained in E.coli NCTC 12390.

c) A reshaped human antibody heavy-chain variable region having specificity for human placental alkaline phosphatase, producible by means of the expression vector contained in E.coli NCTC 12389.

d) A reshaped human antibody light-chain variable region having specificity for human placental alkaline

phosphatase, producible by means of the expression vector contained in E.coli NCTC 12390.

e) A reshaped human antibody or reshaped human antibody fragment, comprising at least one variable region according to c) or d) above.

A particular embodiment of the invention is therefore a reshaped human antibody or fragment possessing anti-PLAP specificity and incorporating a combination of CDRs (which may, for example, be cloned from a murine anti-PLAP immunoglobulin) having the amino acid sequences identified as CDR1, CDR2 and CDR3 respectively in Figures 1 and 2 of the accompanying drawings, which respectively represent the heavy chain variable region (VH) and light chain variable region (Vk) of a murine anti-PLAP monoclonal antibody that we have cloned and sequenced. In the case of an intact antibody, or a fragment comprising at least one heavy chain variable region and at least one light chain variable region, the reshaped antibody or fragment preferably contains all six CDRs from the non-human source. To be most effective in binding, the CDRs should preferably be sited relative to one another in the same arrangement as occurs in the original non-human antibody, e.g. the VH CDRs should be in a human VH framework, and in the order in which they occur naturally in the non-human antibody.

As will be apparent to those skilled in the art, the CDR sequences and the surrounding framework sequences can be subject to minor modifications and variations without the essential specific binding capability being significantly reduced. Such minor modifications and variations can be present either at the genetic level or in the amino acid sequence, or both. Accordingly, the

invention encompasses synthetic (reshaped) antibodies and fragments that are functionally equivalent to those described herein having precisely defined genetic or amino acid sequences.

The invention can also be applied in the production of bi-specific antibodies, having two Fab portions of different specificity, wherein one of the specificities is conferred by a reshaped human variable chain region incorporating one or more of the CDRs depicted in Figures 1 and 2 of the accompanying drawings.

The invention can also be applied in the production of so-called single-chain antibodies (for example, as disclosed in Genex EP-A-281604), and also to polysaccharide-linked antibodies (see Hybritech EP-A-315456) and other modified antibodies.

Any human constant regions (for example, gamma 1, 2, 3 or 4-type) can be used.

Antibody fragments retaining useful specific binding properties can be $(Fab)_2$, Fab, Fv, VH or Vk fragments. These can be derived from an intact reshaped antibody, for example by protease digestion, or produced as such by genetic engineering.

Practical applications of the invention

An important aspect of the invention is a reshaped human anti-PLAP antibody or fragment, as defined above linked to or incorporating an agent capable of retarding or terminating the growth of cancerous cells, or to an imaging agent capable of being detected while inside the human body. The invention also includes injectable

compositions comprising either of such combinations in a pharmaceutically acceptable carrier, such as saline solution, plasma extender or liposomes. The invention also includes the use, in a method of human cancer therapy or imaging, of a reshaped human anti-PLAP antibody or fragment as defined above. The invention further includes the use of such an antibody or fragment for the manufacture of a medicament for therapeutic application in the relief of cancer in humans, or the use of such an antibody or fragment in the manufacture of a diagnostic composition for in-vivo diagnostic application in humans.

The Fc region of the antibody, itself using pathways and mechanisms available in the body, such as complement lysis and antibody dependent cellular cytotoxicity, can be used to affect adversely the growth of cancerous cells. In this embodiment, no additional reagent need be linked to the reshaped antibody.

Examples of agents capable of affecting adversely the growth of cancerous cells include radioisotopes, such as Yttrium 90 and Iodine 131; drugs such as methotrexate; toxins such as ricin or parts thereof; and enzymes which may for example turn an inactive drug into an active drug at the site of antibody binding.

Examples of imaging agents include radioisotopes generating gamma rays, such as Indium 111 and Technetium 99; radioisotopes generating positrons, such as Copper 64; and passive agents such as Barium which act as contrast agents for X-rays, and Gadolinium in nmr/esr scanning.

In order to link a metallic agent, such as a radioisotope, to a specific binding agent of the invention, it may be necessary to employ a coupling or

chelating agent. Many suitable chelating agents have been developed, and reference can be made for example to US 4824986, US 4831175, US 4923985 and US 4622420. Techniques involving the use of chelating agents are described, for example, in US 4454106, US 4722892, Moi et al (1988), McCall et al (1990), Deshpande et al (1990) and Meares et al (1990).

The use of radiolabelled antibodies and fragments in cancer imaging and therapy in humans is described for example in EP 35265. It may be advantageous to use the radiolabelled cancer-specific antibody or fragment in conjunction with a non-specific agent radiolabelled with a different isotope, to provide a contrasting background for so-called subtraction imaging.

The antibody reagents of the invention can be used to identify, e.g. by serum testing or imaging, and/or to treat, PLAP-producing cancers. Such cancers can occur as, for example, breast cancer, ovarian cancer and colon cancer, or can manifest themselves as liquids such as pleural effusions.

Modified antibody production

The portions of the VH and VL regions that by convention (Kabat, 1987) are designated as being the CDRs may not be the sole features that need to be transferred from the non-human monoclonal antibody. Sometimes, enhanced antibody performance, in terms of specificity and/or affinity, can be obtained in the reshaped human antibody if certain non-human framework sequences are conserved in the reshaped human antibody. The objective is to conserve the important three-dimensional protein

structure associated with the CDRs, which is supported by contacts with framework residues.

The normal starting point from which a reshaped antibody in accordance with the invention can be prepared, is a cell (preferably an immortalised cell line), derived from a non-human host animal (for example, a mouse), which expresses an antibody having specificity against human PLAP. Such a cell line can, for example, be a hybridoma cell line prepared by conventional monoclonal antibody technology. Preferably, the expressed antibody has a high affinity and high specificity for PLAP, because it should be anticipated that some loss of affinity and/or specificity may occur during the transfer of these properties to a human antibody or fragment by the procedures of the invention. By selecting a high affinity and high specificity antibody as the parent antibody, the likelihood that the final reshaped antibody or fragment will also exhibit effective binding properties is enhanced.

The next stage is the cloning of the cDNA from the cell expressing the selected non-human antibody, and sequencing and identification of the variable region genes including the sequences encoding the CDRs. The procedures involved can now be regarded as routine in the art, although they are still laborious.

If the object is to produce a reshaped complete human antibody, or at least a fragment of such an antibody which will contain both heavy and light variable domains, it will be necessary to sequence the cDNA associated with both of these domains.

Once the relevant cDNA sequence or sequences have been analysed, it is necessary to prepare one or more replicable expression vectors containing a DNA sequence which encodes at least a variable domain of an antibody, which variable domain comprises human framework regions together with one or more CDRs derived from the selected non-human anti-PLAP antibody. The DNA sequence in each vector should include appropriate regulatory sequences necessary to ensure efficient transcription and translation of the gene, particularly a promoter and leader sequence operably linked to the variable domain sequence. In a typical procedure to produce a reshaped antibody or fragment in accordance with the invention, it may be necessary to produce two such expression vectors, one containing a DNA sequence for a reshaped human light chain and the other, a DNA sequence for a reshaped human heavy chain. The expression vectors should be capable of transforming a chosen cell line in which the production of the reshaped antibody or fragment will occur. Such a cell line may be, for example, a stable non-producing myeloma cell line, examples (such as NS0 and sp2-0) of which are readily available commercially. An alternative is to use a bacterial system, such as E.coli, as the expression vehicle for the reshaped antibody or fragment. The final stages of the procedure therefore involve transforming the chosen cell line or organism using the expression vector or vectors, and thereafter culturing the transformed cell line or organism to yield the reshaped human antibody or fragment.

By way of example only, detailed steps by means of which appropriate expression vectors can be prepared are given later in this specification. The manipulation of DNA material in a suitably equipped laboratory is now a well-developed art, and the procedures required are well

within the skill of those versed in this art. Many appropriate genomic and cDNA libraries, plasmids, restriction enzymes, and the various reagents and media which are required in order to perform such manipulations, are available commercially from suppliers of laboratory materials. For example, genomic and cDNA libraries can be purchased from Clontech Laboratories Inc. The steps given by way of example below are purely for the guidance of the reader of this specification, and the invention is in no way critically dependant upon the availability of one or more special starting materials. In practice, the skilled person has a wide range of materials from which to choose, and can exploit and adapt the published technology using acquired experience and materials that are most readily available in the scientific environment. For example, many plasmids fall into this category, having been so widely used and circulated within the relevant scientific community that they can now be regarded as common-place materials.

Examples

The procedure used to prepare reshaped anti-PLAP human antibodies is described in detail below, by way of example only, with reference to the accompanying drawings, of which:

Figure 1 shows the cDNA sequence coding for a murine heavy chain variable region having anti-PLAP specificity. The 3 classical CDRs are indicated, together with an amino acid sequence matching the cDNA code.

Figure 2 shows the cDNA sequence coding for a murine light chain variable region having anti-PLAP specificity.

Figures 3a and 3b together show a route by which an expression vector encoding a reshaped human heavy chain incorporating the CDRs of Figure 1, can be prepared.

Figures 4a and 4b together show a similar transformation route to obtain an expression vector encoding a reshaped human light chain incorporating the CDRs of Figure 2, can be prepared.

Figure 5 shows the plasmid pU12-IgEnh, which contains an enhancer sequence used in the routes of Figures 4a and 4b.

Figure 6 shows the source of plasmid pBGS18-HulgG1 used in the route of Figure 3b.

Figure 7 shows the source of plasmid pBGS18-HuCk used in the route of Figure 4b.

Figure 8 shows two synthetic oligonucleotide sequences I and II used in cloning the cDNA sequences of Figures 1 and 2.

Figure 9 shows six synthetic oligonucleotide sequences III to VIII used in the routes depicted in Figures 3a-4b.

Figures 10 and 11 show the cDNA and amino acid sequences of the resulting reshaped human heavy and light chain variable regions respectively.

Figure 12 shows in graphical form the relative specific anti-PLAP binding activity of the resulting reshaped human antibody.

Figure 13 depicts in diagrammatic form the structure of a typical antibody (immunoglobulin) molecule.

The experimental procedures required to practice the invention do not in themselves represent unusual technology, and they involve straightforward cloning and mutagenesis techniques as generally described for example in Verhoeyen et al (1988); Riechmann et al (1988) and EP-A-239400 (Winter). Alternatively, if an appropriate DNA sequence is already known in detail (the drawings accompanying this specification includes a sequence associated with anti-PLAP specificity), the reshaped human variable region genes can be synthesised in vitro (see Jones et al, 1986). Laboratory equipment and reagents for synthesising long oligonucleotides are readily available, and as techniques in this field develop it is becoming practicable to synthesise progressively longer sequences.

Detailed laboratory manuals, covering all basic aspects of recombinant DNA techniques, are available, e.g. "Molecular Cloning" by Sambrook et al (1989).

By means of the invention, the antigen binding regions of a mouse anti-PLAP antibody were grafted onto human framework regions. The resulting reshaped human antibody (designated Hu2PLAP) has binding characteristics similar to those of the original mouse antibody.

Such reshaped antibodies can be used for in vivo diagnosis and treatment of human cancers, eg. ovarian cancers and seminoma, and are expected at least to reduce the problem of an immune response in the patient often seen upon administration of non-human antibody. A similar benefit has been shown for reshaped CAMPATH-1 antibody in Hale et al (1988).

Methods:

a) Cloning and sequence determination of the mouse variable region genes

Messenger RNA was isolated from the murine hybridoma line "H17E2" which secretes a gamma-1, kappa anti-PLAP antibody, described in Travers et al (1984). First strand cDNA was synthesised by priming with oligonucleotides I and II (see Figure 8) complementary to the 5' ends of the CH1 and Ck exons respectively. Second strand cDNA was obtained as described by Gübler and Hoffmann (1983).

Kinased EcoRI linkers were ligated to the now double-stranded cDNA (which was first treated with EcoRI methylase, to protect possible internal EcoRI sites), followed by cloning into EcoRI-cut pUC9 (Vieira et al, 1982) and transformation of E.coli strain TG2 (Gibson, 1984).

Colonies containing genes coding for murine anti-PLAP VH (MoVHPLAP) and for murine anti-PLAP Vk (MoVkPLAP) were identified by colony hybridisation with 2 probes consisting respectively of 32P-labelled first strand cDNA of anti-PLAP VH and Vk. Positive clones were characterised by plasmid preparation, followed by EcoRI digestion and 1.5% agarose gel analysis. Full-size inserts (about 450bp) were subcloned in the EcoRI site of M13mp18 (Norrander et al, 1983). This yielded clones with inserts in both orientations, facilitating nucleotide sequence determination of the entire insert, by the dideoxy chain termination method (Sanger et al, 1977).

The nucleotide sequences, and their translation into amino acid sequences, of the mature variable region genes

MoVHPLAP and MoVkPLAP, are shown in Figures 1 and 2. The 450 bp inserts included a signal sequence and 5' untranslated sequences and linkers, not shown in the Figures.

b) Grafting of the mouse anti-PLAP CDRs onto human framework regions

The general techniques necessary to achieve this have been described very adequately in Jones et al (1986), Verhoeyen et al (1988), Riechmann et al (1988) and in EP-A-239400 (Winter).

The basic constructs used for reshaping were M13mp9HuVHLYS (Verhoeyen et al, 1988) and M13mp9HuVkLYS (Riechmann et al, 1988), which respectively contain the framework regions of the heavy chain variable region of human "NEW" and of the light chain variable region of human "REI". Both of these human antibodies have been thoroughly characterised and reported (Saul et al, 1978; and Epp et al, 1974, respectively).

The CDRs in these constructs (Figures 3a and 4a) were replaced by site-directed mutagenesis with oligonucleotides encoding the anti-PLAP CDRs flanked by at least 12 nucleotides at each end encoding the corresponding human framework residues. These oligonucleotides are shown in Figure 9, in which the sequences corresponding to the CDRs are underlined.

In the present instance we found it useful also to conserve the amino acids Phe 27 and Thr 30 of the murine VHPLAP in the VH domain of the reshaped human anti-PLAP antibody. In oligonucleotide III, with 24 nucleotides

flanking the 5' end of CDR 1, the murine Phe 27 and Thr 30 codons are shown in italics in Figure 9.

The mutagenesis was done as described in Riechmann et al (1988). The resulting variable regions were named Hu2VHPLAP and HuVkPLAP and are shown in Figure 10 and 11.

c) Assembly of reshaped human antibody genes in expression vectors

The next stage involved the use of a murine heavy chain enhancer IgEnh, described in Neuberger et al (1983) where the enhancer is contained in a 1kb Xbal fragment of plasmid pSV-Vμ1. The 700bp Xbal/EcoRI subfragment of this 1kb Xbal fragment is sufficient to confer enhancer activity.

The reshaped human genes as prepared in section (b) above were excised from the M13 vectors as HindIII - BamHI fragments. The heavy chain variable region genes were cloned into a vector based on pSV2gpt (Mulligan et al, 1981), and the light chain variable region genes were cloned into a vector based on pSV2neo (Southern et al, 1981). Both contained the immunoglobulin heavy chain enhancer IgEnh. In the pSV2gpt based antibody expression vector (see Fig. 4b - 4c), the Xbal/EcoRI enhancer containing fragment was cloned in the unique EcoRI site of the pSV2gpt vector (after ligating EcoRI linkers to the filled in Xbal end of the fragment). The vector pSVgptMoVHLYS-MoIgG1 (Verhoeyen et al, 1988) was used as the source of a pSVgpt-based vector containing the IgEnh enhancer.

In the pSVneo based antibody expression vector (see Fig. 5a - 5b), the 1kb Xbal enhancer containing fragment

was first cloned into pUC12 (Vieira et al, 1982), yielding the plasmid pUC12-IgEnh, see Figure 5. The enhancer can then be cut out as a 700bp EcoRI/HindIII fragment (either orientation of the enhancer will work), and cloned in the pSV2neo-derived vector (pSVneoMSN409 as shown in Figure 4a) obtained by removing the HindIII site in pSVneo. It is possible to use pSV2gpt as an alternative vector for light chain expression, as in practice there is no need for neo selection.

The Hu2VHPLAP gene was linked to a human gamma 1 constant region (Takahashi et al, 1982), cloned initially as a 8kb HindIII fragment into the HindIII site of pBGS18 (Spratt et al, 1986), and then in the pSV2gpt expression vector as a BamHI fragment (see Figures 3b and 6). It should be noted that in the Takahashi et al (1982) reference there is an error in Figure 1: the last (3') two sites are BamH1 followed by HindIII, and not the converse. This was confirmed by Flanagan et al (1982).

The HuVkPLAP gene was linked to a human C kappa constant region (Hieter et al, 1980) also cloned in as a BamHI fragment (see Figures 4b and 7). The source of the human Ck used in Figure 7 is given in Hieter et al (1980). The 12 kb BamH1 fragment from embryonic DNA (cloned in a gamma Ch28 vector system) was subcloned in the BamH1 site of plasmid pBR322.

d) Expression in myeloma cells

Co-transfection of the expression plasmids pSVgptHu2VHPLAP-HuIgG1 and pSVneoHuVkPLAP-HuCk (Figures 3b and 4b) into NSO myeloma cells was done by electroporation (Potter et al, 1984), after linearisation with PvuI. Transfectomas were selected in mycophenolic acid

containing medium to select for cells expressing the gpt gene product, and screened for antibody production and anti-PLAP activity by ELISA assays.

Positive clones were subcloned by limiting dilution and pure clones were assayed again for anti-PLAP activity, and the best producing clones were grown in serum-free medium for antibody production.

e) Binding ability of the reshaped human antibodies

The practical application of the reshaped human antibody demands sufficient binding effectiveness. If the parent antibody has a very high effectiveness then some reduction during reshaping can be tolerated. The binding effectiveness will be dictated by many factors, one of which will be the antibody affinity for antigen, in this case placental alkaline phosphatase. A useful way of demonstrating binding ability of the reshaped antibody is to show that it has a similar antibody dilution curve when binding to antigen adsorbed on a plastic well surface. Such curves were generated as follows, using the parent murine anti-PLAP antibody and a reshaped human antibody prepared by the foregoing procedure.

Multiwell plates (Costar 6595, PETG) were coated with placental alkaline phosphatase (5 µg/ml in phosphate buffered saline pH 7.4, 37°C, 2 hours). The plates were rinsed in phosphate buffered saline before blocking with gelatin (0.02% in phosphate buffered saline) for one hour at room temperature, then washed four times with phosphate buffered saline with added Tween 20 (0.15%), and then used.

Antibody binding was performed in phosphate buffered saline with Tween 20 at room temperature for one hour, followed by four washes in buffer.

Visualisation of bound antibody was with horse radish peroxidase conjugated anti-globulins (anti-human IgG for the reshaped antibody and anti-mouse IgG for the parent molecule). The conjugate (Sigma) in buffer (1:1000) was incubated for one hour at room temperature, followed by four washes as above. Colour development (45 minutes) was with tetramethyl benzidene (0.01%) and hydrogen peroxide (1:200 or 100 vols) in citrate buffer pH6.5. The reaction was stopped with 2M hydrochloric acid.

Controls showed insignificant colour due to non-specific binding of conjugate or due to binding of antibody to wells not containing placental alkaline phosphatase. The results, shown in Figure 12, are expressed as a percentage of the maximum colour (binding) seen. The two curves are similar, indicating a significant and useful level of binding effectiveness for the reshaped antibody of the invention.

f) Deposited plasmids

E.coli strains containing plasmids used in the above procedure have been deposited, in accordance with the provisions of the Budapest Treaty, in the National Collection of Type Cultures on 19 April 1990 as follows:

NCTC 12389: K12, TG1 E.coli containing plasmid pSVgptHu2VHPLAP-HuIgG1

NCTC 12390: K12, TG1 E.coli containing plasmid pSVneoHuVkPLAP-HuCk

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CLAIMS

1. A synthetic specific binding agent having specificity for human placental alkaline phosphatase.

2. A synthetic specific binding agent according to claim 1, wherein the anti-PLAP specificity is conferred by the presence of one or more of the amino acid sequences:

i) Ser Tyr Gly Val Ser

ii) Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His Ser Ala Leu Ile Ser

iii) Pro His Tyr Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr

iv) Arg Ala Ser Glu Asn Ile Tyr Ser Tyr Val Ala

v) Asn Ala Lys Ser Leu Ala Glu

vi) Gln His His Tyr Val Ser Pro Trp Thr

3. A reshaped human antibody, or a reshaped human antibody fragment, having specificity for human placental alkaline phosphatase.

4. A reshaped human antibody or reshaped human antibody fragment according to claim 3, wherein the anti-PLAP specificity is conferred by the presence of one or more of the amino acid sequences:

i) Ser Tyr Gly Val Ser

ii) Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His Ser Ala Leu Ile Ser

iii) Pro His Tyr Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr

iv) Arg Ala Ser Glu Asn Ile Tyr Ser Tyr Val Ala

v) Asn Ala Lys Ser Leu Ala Glu

vi) Gln His His Tyr Val Ser Pro Trp Thr

5. A reshaped human antibody or reshaped human antibody fragment according to claim 3, having at least one heavy-chain variable region incorporating the following CDRs:

CDR1: Ser Tyr Glu Val Ser

CDR2: Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His Ser Ala Leu Ile Ser

CDR3: Pro His Tyr Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr

6. A reshaped human antibody or reshaped human antibody fragment according to claim 3, having at least one light-chain variable region incorporating the following CDRs:

CDR1: Arg Ala Ser Gly Asn Ile Tyr Ser Tyr Val Ala

CDR2: Asn Ala Lys Ser Leu Ala Glu

CDR3: Gln His His Tyr Val Ser Pro Trp Thr

7. A reshaped human antibody or reshaped human antibody fragment according to claim 3 and having at least one heavy-chain variable region according to claim 5 and at

least one light-chain variable region according to claim 6.

8. A reshaped human antibody or reshaped human antibody fragment according to claim 3, incorporating at least one heavy-chain variable region comprising the entire amino acid sequence depicted in Figure 10 of the accompanying drawings.

9. A reshaped human antibody or reshaped human antibody fragment according to claim 3, incorporating at least one light-chain variable region comprising the entire amino acid sequence depicted in Figure 11 of the accompanying drawings.

10. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, having specificity equivalent to that of the gamma-1, kappa anti-PLAP monoclonal antibody secreted by murine hybridoma cell line H17E2.

11. A stable host cell line producing a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment according to any one of claims 1 to 10, resulting from incorporation in the cell line of a foreign gene encoding the synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

12. A stable host cell line according to claim 11, wherein the foreign gene includes one or more of the nucleotide sequences:

i) AGT TAT GGT GTA AGC

ii) GTA ATA TGG GAA GAC GGG AGC ACA AAT TAT CAT TCA GCT CTC ATA TCC

iii) CCC CAC TAC GGT AGC AGC TAC GTG GGG GCT ATG GAA TAC

iv) CGA GCA AGT GAA AAT ATT TAC AGT TAT GTA GCA

v) AAT GCA AAA TCC TTA GCA GAG

vi) CAA CAT CAT TAT GTT AGT CCG TGG ACG

13. A stable host cell line according to claim 11, wherein the foreign gene includes the entire nucleotide sequence depicted in Figure 10 of the accompanying drawings.

14. A stable host cell line according to claim 11, wherein the foreign gene includes the entire nucleotide sequence depicted in Figure 11 of the accompanying drawings.

15. A stable host cell line according to claim 11, wherein the foreign gene encodes:

a) at least one of the amino acid sequences:

i) Ser Tyr Gly Val Ser

ii) Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His Ser Ala Leu Ile Ser

iii) Pro His Tyr Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr

iv) Arg Ala Ser Glu Asn Ile Tyr Ser Tyr Val Ala

v) Asn Ala Lys Ser Leu Ala Glu

vi) Gln His His Tyr Val Ser Pro Trp Thr

and b) a protein framework that enables the encoded amino acid sequence when expressed to function as a CDR having specificity for human placental alkaline phosphatase.

16. A stable host cell line according to claim 11, wherein the foreign gene encodes the entire amino acid sequence depicted in Figure 10 of the accompanying drawings.

17. A stable host cell line according to claim 11, wherein the foreign gene encodes the entire amino acid sequence depicted in Figure 11 of the accompanying drawings.

18. Plasmid pSVgptHu2VHPLAP-HuIgG1.

19. Plasmid pSVneoHuVkPLAP-HuCk.

20. Use of plasmid according to claim 18 or claim 19 in the production of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

21. E.coli NCTC 12389.

22. E.coli NCTC 12390.

23. A DNA sequence encoding a reshaped human antibody heavy-chain variable region having specificity for human placental alkaline phosphatase, as contained in E.coli NCTC 12389.

24. A DNA sequence encoding a reshaped human antibody light-chain variable region having specificity for human placental alkaline phosphatase, as contained in E.coli NCTC 12390.

25. A reshaped human antibody heavy-chain variable region having specificity for human placental alkaline phosphatase, producible by means of the expression vector contained in E.coli NCTC 12389.

26. A reshaped human antibody light-chain variable region having specificity for human alkaline phosphatase, producible by means of the expression vector contained in E.coli NCTC 12390.

27. A reshaped human antibody or reshaped human antibody fragment, comprising at least one variable region according to claim 25 or claim 26.

28. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of claims 1 to 10 or claim 27, linked to or incorporating an agent capable of retarding or terminating the growth of cancerous cells, or linked to an agent capable of being detected while inside the human body.

29. An injectable composition comprising a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to claim 28, in a pharmaceutically acceptable carrier.

30. Use of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of claims 1 to 10 or claim 27, for the manufacture of a medicament for therapeutic

application in the relief of cancer in humans, or for the manufacture of a diagnostic composition for in-vivo diagnostic application in humans.

31. Use of a synthetic binding agent, reshaped human antibody or reshaped human antibody fragment, according to claim 28, in a method of human cancer therapy or imaging.

AMEMDED CLAIMS

[received by the International Bureau
on 07 May 1991 (07.05.91);
original claims 1 and 2 replaced by new claim 1;
claims 3 and 4 replaced by new claim 2;
claims 5-31 unchanged but renumbered as claims 3-29 (6 pages)]

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1. A synthetic specific binding agent having specificity for human placental alkaline phosphatase conferred by the presence of one or more of the amino acid sequences:

i) Ser Tyr Gly Val Ser

ii) Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His Ser Ala Leu Ile Ser

iii) Pro His Tyr Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr

iv) Arg Ala Ser Glu Asn Ile Tyr Ser Tyr Val Ala

v) Asn Ala Lys Ser Leu Ala Glu

vi) Gln His His Tyr Val Ser Pro Trp Thr

2. A reshaped human antibody, or a reshaped human antibody fragment, having specificity for human placental alkaline phosphatase conferred by the presence of one or more of the amino acid sequences:

i) Ser Tyr Gly Val Ser

ii) Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His Ser Ala Leu Ile Ser

iii) Pro His Tyr Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr

iv) Arg Ala Ser Glu Asn Ile Tyr Ser Tyr Val Ala

v) Asn Ala Lys Ser Leu Ala Glu

vi) Gln His His Tyr Val Ser Pro Trp Thr

3. A reshaped human antibody or reshaped human antibody fragment according to claim 2, having at least one heavy-chain variable region incorporating the following CDRs:

CDR1: Ser Tyr Gly Val Ser

CDR2: Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His Ser Ala Leu Ile Ser

CDR3: Pro His Tyr Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr

4. A reshaped human antibody or reshaped human antibody fragment according to claim 2, having at least one light-chain variable region incorporating the following CDRs:

CDR1: Arg Ala Ser Glu Asn Ile Tyr Ser Tyr Val Ala

CDR2: Asn Ala Lys Ser Leu Ala Glu

CDR3: Gln His His Tyr Val Ser Pro Trp Thr

5. A reshaped human antibody or reshaped human antibody fragment according to claim 2 and having at least one heavy-chain variable region according to claim 3 and at least one light-chain variable region according to claim 4.

6. A reshaped human antibody or reshaped human antibody fragment according to claim 2, incorporating at least one heavy-chain variable region comprising the entire amino

acid sequence depicted in Figure 10 of the accompanying drawings.

7. A reshaped human antibody or reshaped human antibody fragment according to claim 2, incorporating at least one light-chain variable region comprising the entire amino acid sequence depicted in Figure 11 of the accompanying drawings.

8. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, having specificity equivalent to that of the gamma-1, kappa anti-PLAP monoclonal antibody secreted by murine hybridoma cell line H17E2.

9. A stable host cell line producing a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment according to any one of claims 1 to 8, resulting from incorporation in the cell line of a foreign gene encoding the synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

10. A stable host cell line according to claim 9, wherein the foreign gene includes one or more of the nucleotide sequences:

i) AGT TAT GGT GTA AGC

ii) GTA ATA TGG GAA GAC GGG AGC ACA AAT TAT CAT TCA GCT CTC ATA TCC

iii) CCC CAC TAC GGT AGC AGC TAC GTG GGG GCT ATG GAA TAC

iv) CGA GCA AGT GAA AAT ATT TAC AGT TAT GTA GCA

v) AAT GCA AAA TCC TTA GCA GAG

vi) CAA CAT CAT TAT GTT AGT CCG TGG ACG

11. A stable host cell line according to claim 9, wherein the foreign gene includes the entire nucleotide sequence depicted in Figure 10 of the accompanying drawings.

12. A stable host cell line according to claim 9, wherein the foreign gene includes the entire nucleotide sequence depicted in Figure 11 of the accompanying drawings.

13. A stable host cell line according to claim 9, wherein the foreign gene encodes:

a) at least one of the amino acid sequences:

i) Ser Tyr Gly Val Ser

ii) Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His Ser Ala Leu Ile Ser

iii) Pro His Tyr Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr

iv) Arg Ala Ser Glu Asn Ile Tyr Ser Tyr Val Ala

v) Asn Ala Lys Ser Leu Ala Glu

vi) Gln His His Tyr Val Ser Pro Trp Thr

and b) a protein framework that enables the encoded amino acid sequence when expressed to function as a CDR having specificity for human placental alkaline phosphatase.

14. A stable host cell line according to claim 9, wherein the foreign gene encodes the entire amino acid sequence depicted in Figure 10 of the accompanying drawings.

15. A stable host cell line according to claim 9, wherein the foreign gene encodes the entire amino acid sequence depicted in Figure 11 of the accompanying drawings.

16. Plasmid pSVgptHu2VHPLAP-HuIgG1.

17. Plasmid pSVneoHuVkPLAP-HuCk.

18. Use of plasmid according to claim 16 or claim 17 in the production of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

19. E.coli NCTC 12389.

20. E.coli NCTC 12390.

21. A DNA sequence encoding a reshaped human antibody heavy-chain variable region having specificity for human placental alkaline phosphatase, as contained in E.coli NCTC 12389.

22. A DNA sequence encoding a reshaped human antibody light-chain variable region having specificity for human placental alkaline phosphatase, as contained in E.coli NCTC 12390.

23. A reshaped human antibody heavy-chain variable region having specificity for human placental alkaline phosphatase, producible by means of the expression vector contained in E.coli NCTC 12389.

24. A reshaped human antibody light-chain variable region having specificity for human alkaline phosphatase, producible by means of the expression vector contained in E.coli NCTC 12390.

25. A reshaped human antibody or reshaped human antibody fragment, comprising at least one variable region according to claim 23 or claim 24.

26. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of claims 1 to 8or claim 25, linked to or incorporating an agent capable of retarding or terminating the growth of cancerous cells, or linked to an agent capable of being detected while inside the human body.

27. An injectable composition comprising a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to claim 26, in a pharmaceutically acceptable carrier.

28. Use of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of claims 1 to 8 or claim 25, for the manufacture of a medicament for therapeutic application in the relief of cancer in humans, or for the manufacture of a diagnostic composition for in-vivo diagnostic application in humans.

29. Use of a synthetic binding agent, reshaped human antibody or reshaped human antibody fragment, according to claim 26, in a method of human cancer therapy or imaging.

Fig.1.

MoVHPLAP

```
                    5                  10                  15                  20
CAG GTG CAG CTG AAG GAG TCA GGA CCT GGC CTG GTG GCG CCC TCA CAG AGC CTG TCC ATC     60
Gln Val Gln Leu Lys Glu Ser Gly Pro Gly Leu Val Ala Pro Ser Gln Ser Leu Ser Ile

                    25                  30        CDR1      35                  40
ACA TGC ACT GTC TCA GGG TTC TCA TTA ACC AGT TAT GGT GTA AGC TGG GTT CGC CAG CCT    120
Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Ser Tyr Gly Val Ser Trp Val Arg Gln Pro

                    45                  50                  55       CDR 2      60
CCA AGA AAG GGT CTG GAG TGG CTG GGA GTA ATA TGG GAA GAC GGG AGC ACA AAT TAT CAT    180
Pro Arg Lys Gly Leu Glu Trp Leu Gly Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His

                    65                  70                  75                  80
TCA GCT CTC ATA TCC AGA CTG AGC ATC AAC AAG GAT AAC TCC AAG AGC CAA GTT TTC TTA    240
Ser Ala Leu Ile Ser Arg Leu Ser Ile Asn Lys Asp Asn Ser Lys Ser Gln Val Phe Leu

     82  A   B   C  83      85                  90                  95      CDR3
AAA CTG AAC AGT CTG CAA ACT GAT GAC ACA GCC ACG TAC TAC TGT GCC AAA CCC CAC TAC    300
Lys Leu Asn Ser Leu Gln Thr Asp Asp Thr Ala Thr Tyr Tyr Cys Ala Lys Pro His Tyr

          100  A   B   C   D   E  101                 105                 110
GGT AGC AGC TAC GTG GGG GCT ATG GAA TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC    360
Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser

TCA                                                                               363
Ser
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Fig. 2.

MoVkPLAP

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                    5                  10                  15                  20
GAC ATC CAG ATG ACT CAG TCT CCA GCC TCC CTA ACT GCA TCT GTG GGA GAA ACT GTC ACC     60
Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Thr Ala Ser Val Gly Glu Thr Val Thr

                   25     CDR 1        30                  35                  40
ATC ACC TGT CGA GCA AGT GAA AAT ATT TAC AGT TAT GTA GCA TGG TAT CAG CAG AAA CAG    120
Ile Thr Cys Arg Ala Ser Glu Asn Ile Tyr Ser Tyr Val Ala Trp Tyr Gln Gln Lys Gln

                   45                  50     CDR 2        55                  60
GGA AAA TCT CCT CAG TTC CTG GTC TAT AAT GCA AAA TCC TTA GCA GAG GGT GTG CCA TCA    180
Gly Lys Ser Pro Gln Phe Leu Val Tyr Asn Ala Lys Ser Leu Ala Glu Gly Val Pro Ser

                   65                  70                  75                  80
AGG TTC AGT GGC AGT GGA TCA GGC ACA CAG TTT TCT CTG AAG ATC AAC AGC CTG CAG CCT    240
Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln Phe Ser Leu Lys Ile Asn Ser Leu Gln Pro

                   85                  90     CDR 3        95                  100
GAA GAT TTT GGG AAT TAT TAC TGT CAA CAT CAT TAT GTT AGT CCG TGG ACG TTC GGT GGA    300
Glu Asp Phe Gly Asn Tyr Tyr Cys Gln His His Tyr Val Ser Pro Trp Thr Phe Gly Gly

                  105
GGC ACC AAG CTG GAA ATC AGA CGG                                                   324
Gly Thr Lys Leu Glu Ile Arg Arg
```

Fig. 3a

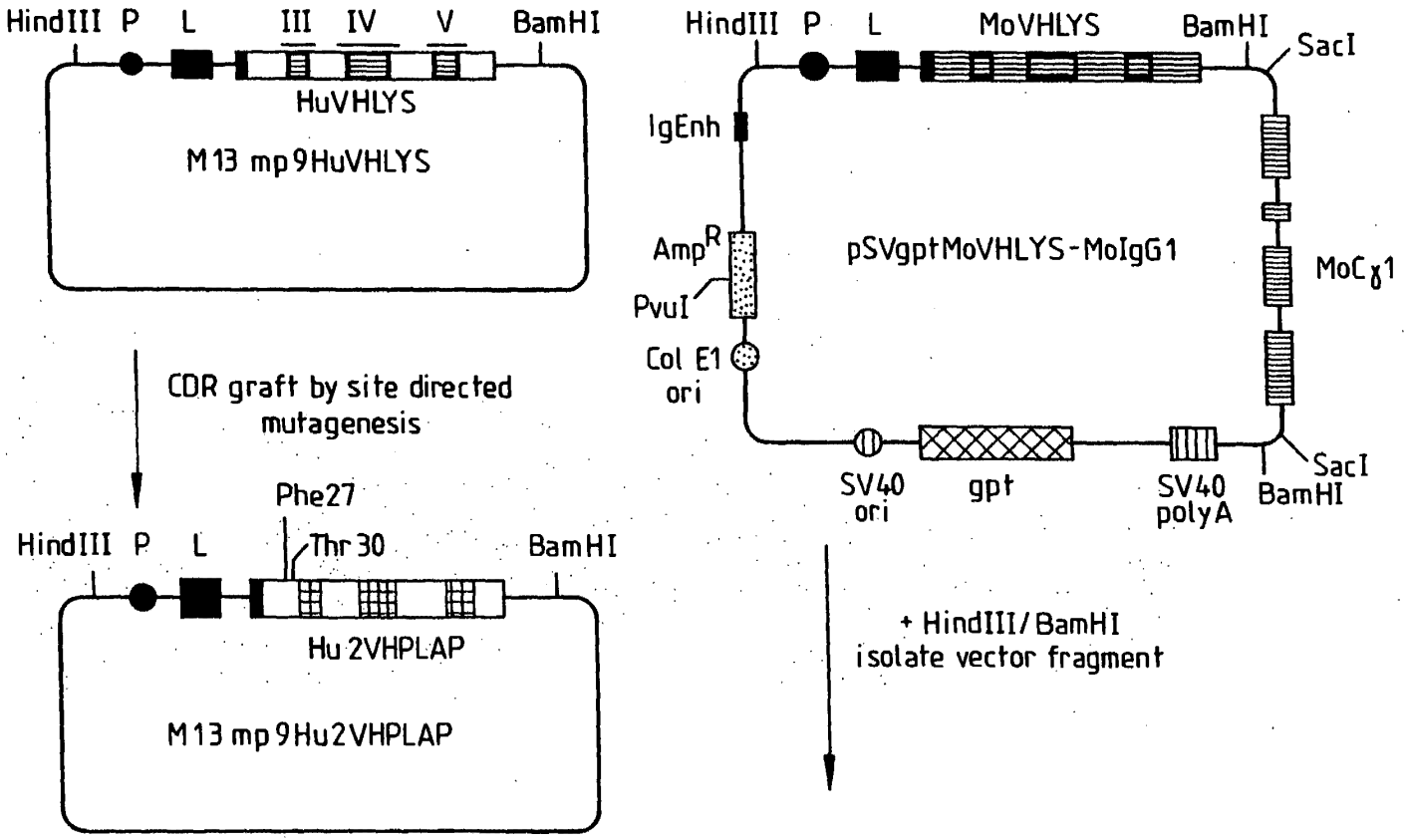
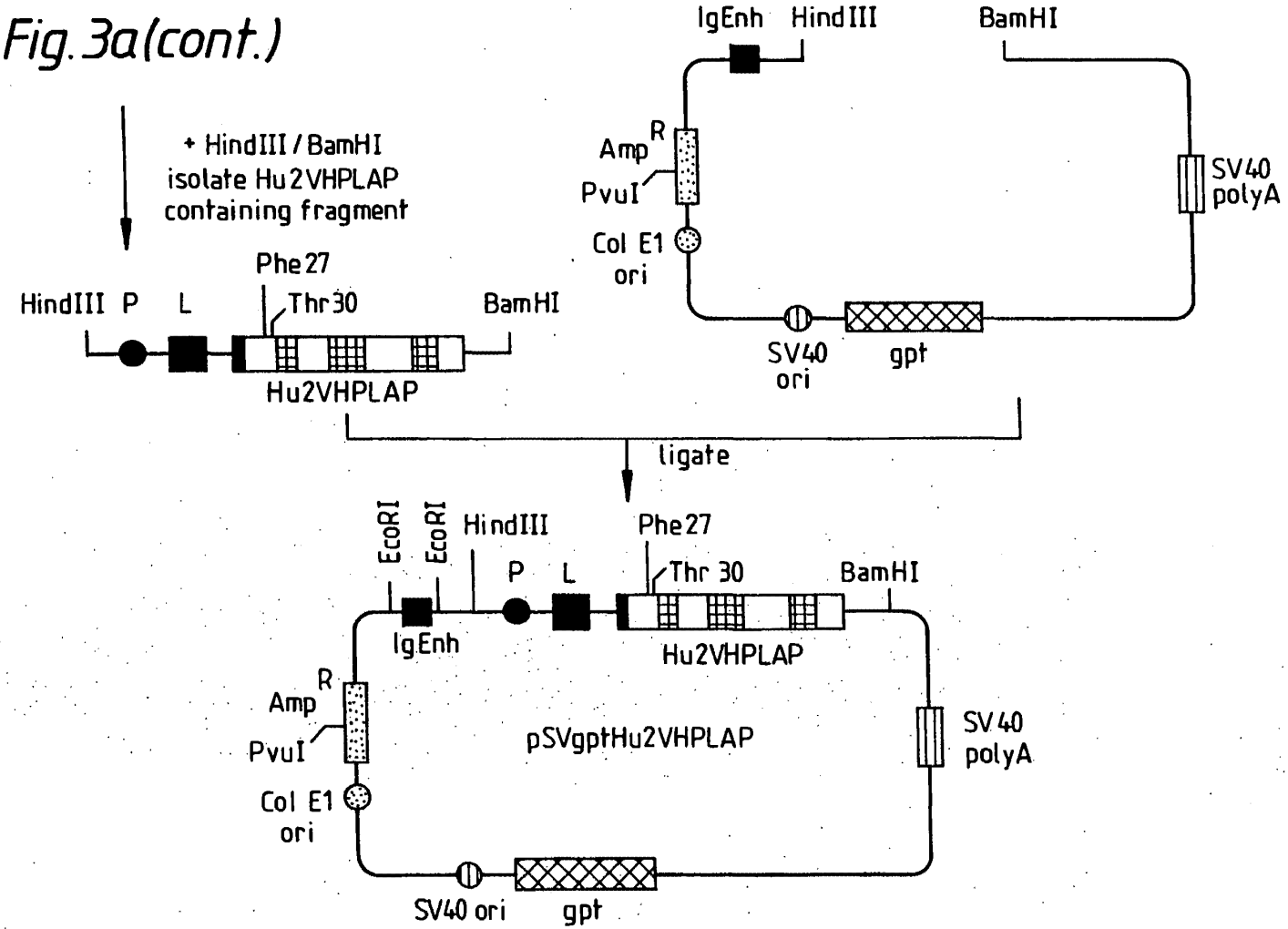


Fig. 3a(cont.)


+ HindIII / BamHI
isolate Hu2VHPLAP
containing fragment
HindIII
P
L
Phe27
Thr30
BamHI
Hu2VHPLAP
IgEnh
HindIII
BamHI
Amp R
PvuI
Col E1
ori
SV40
polyA
SV40
ori
gpt
ligate
EcoRI
EcoRI
HindIII
P
L
Phe27
Thr 30
BamHI
IgEnh
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polyA
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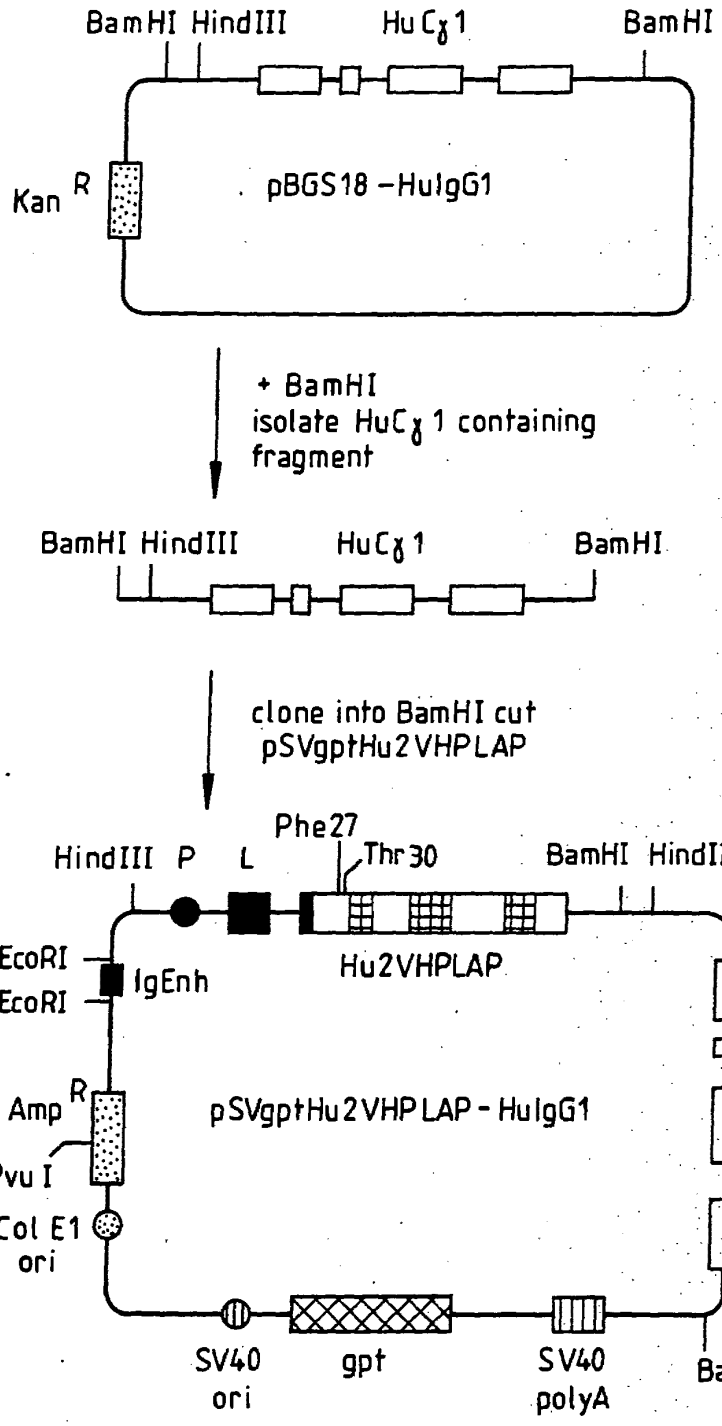


Fig. 3b

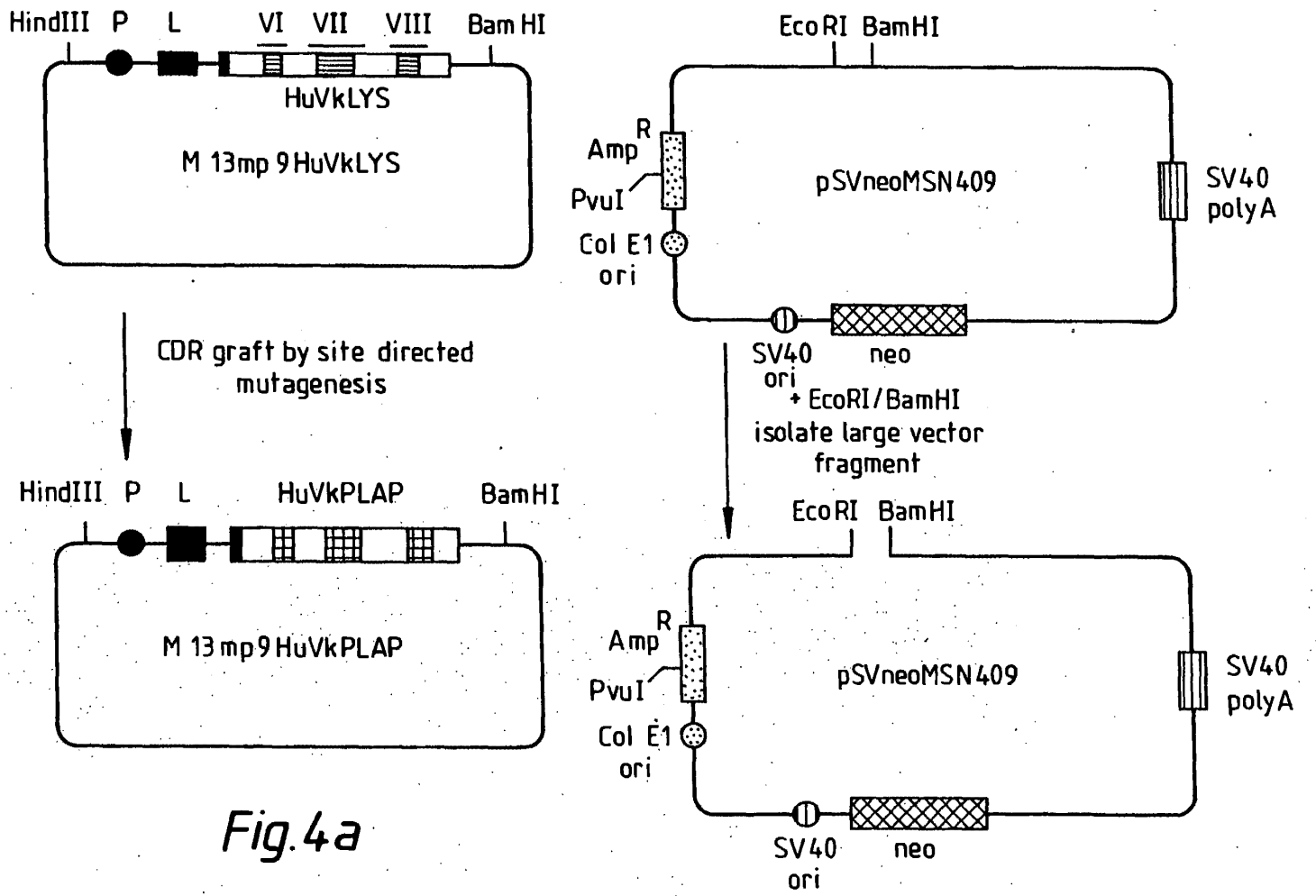
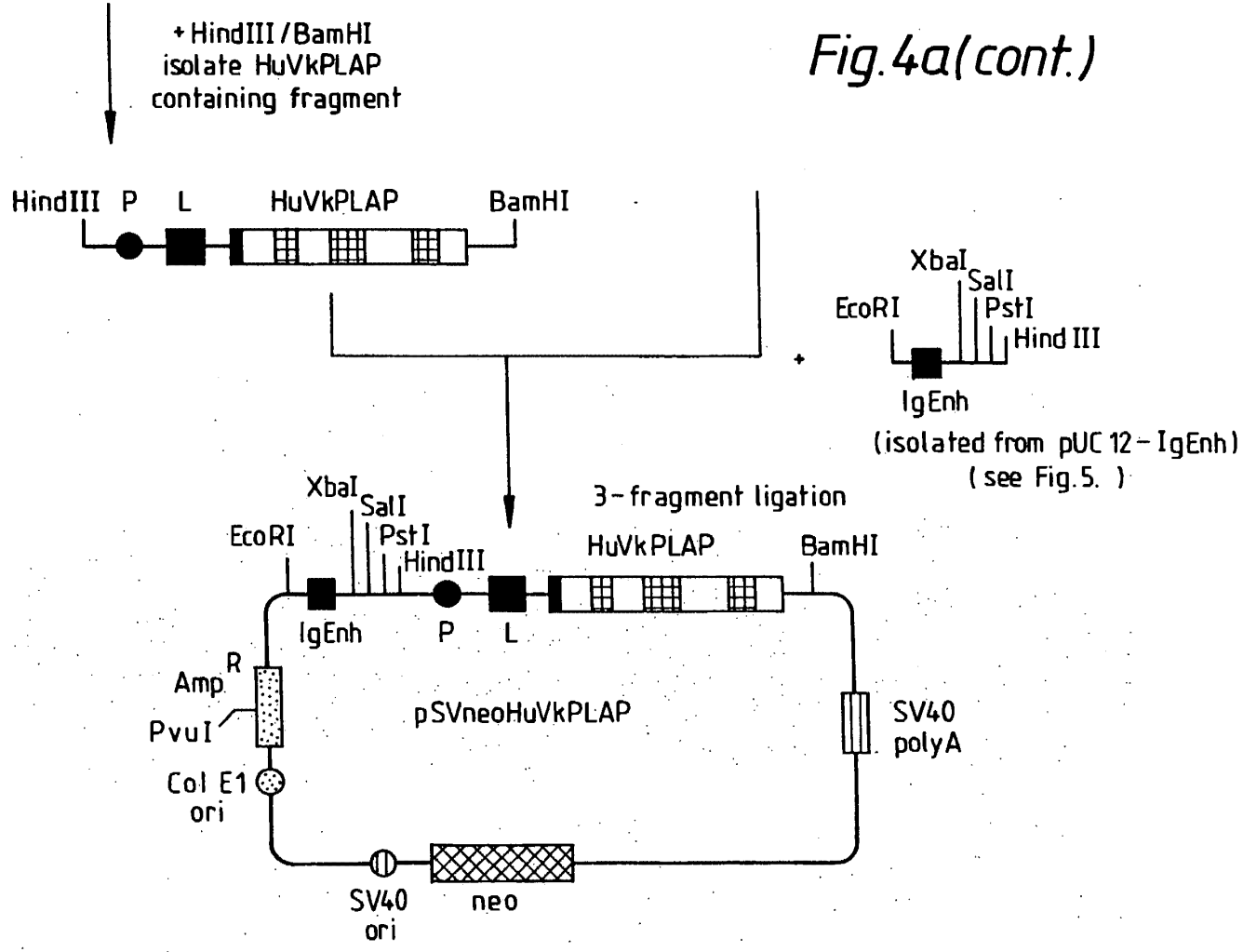


Fig.4a

Fig.4a(cont.)



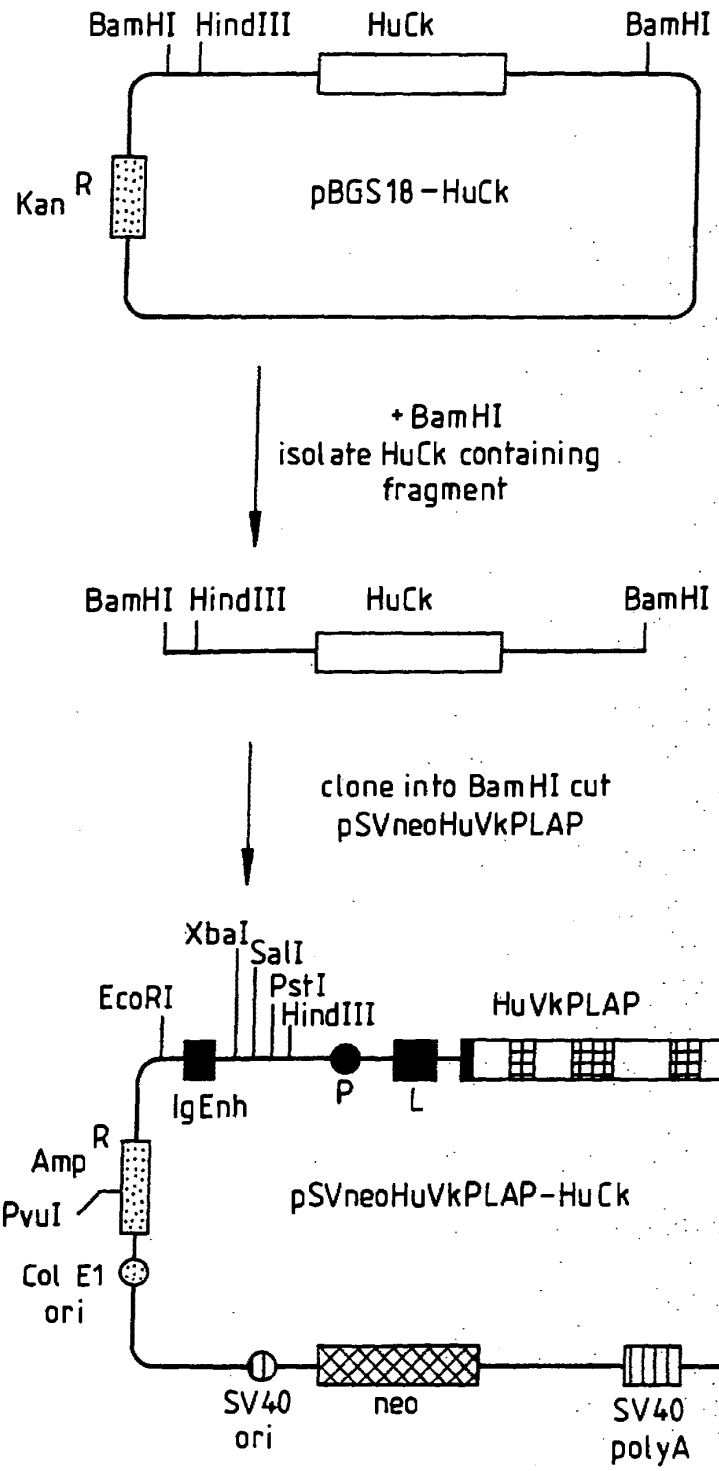


Fig. 4b

Fig. 5.

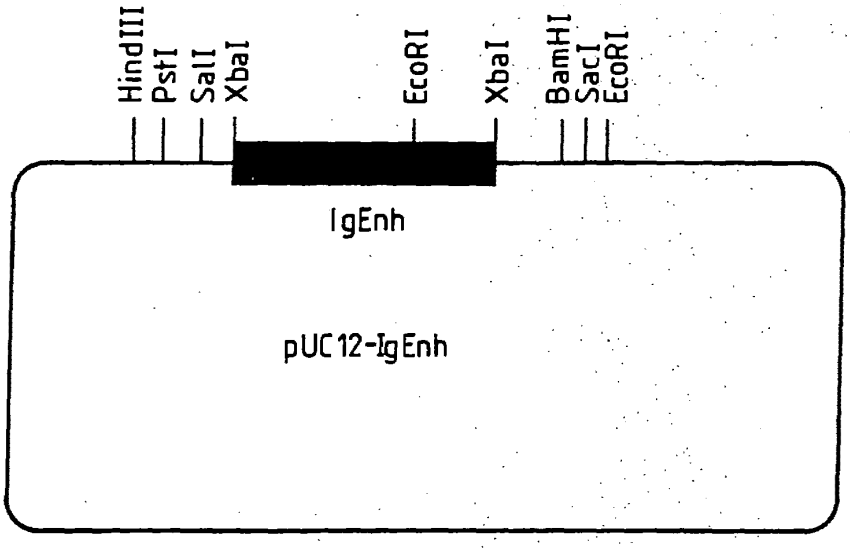


Fig. 6.

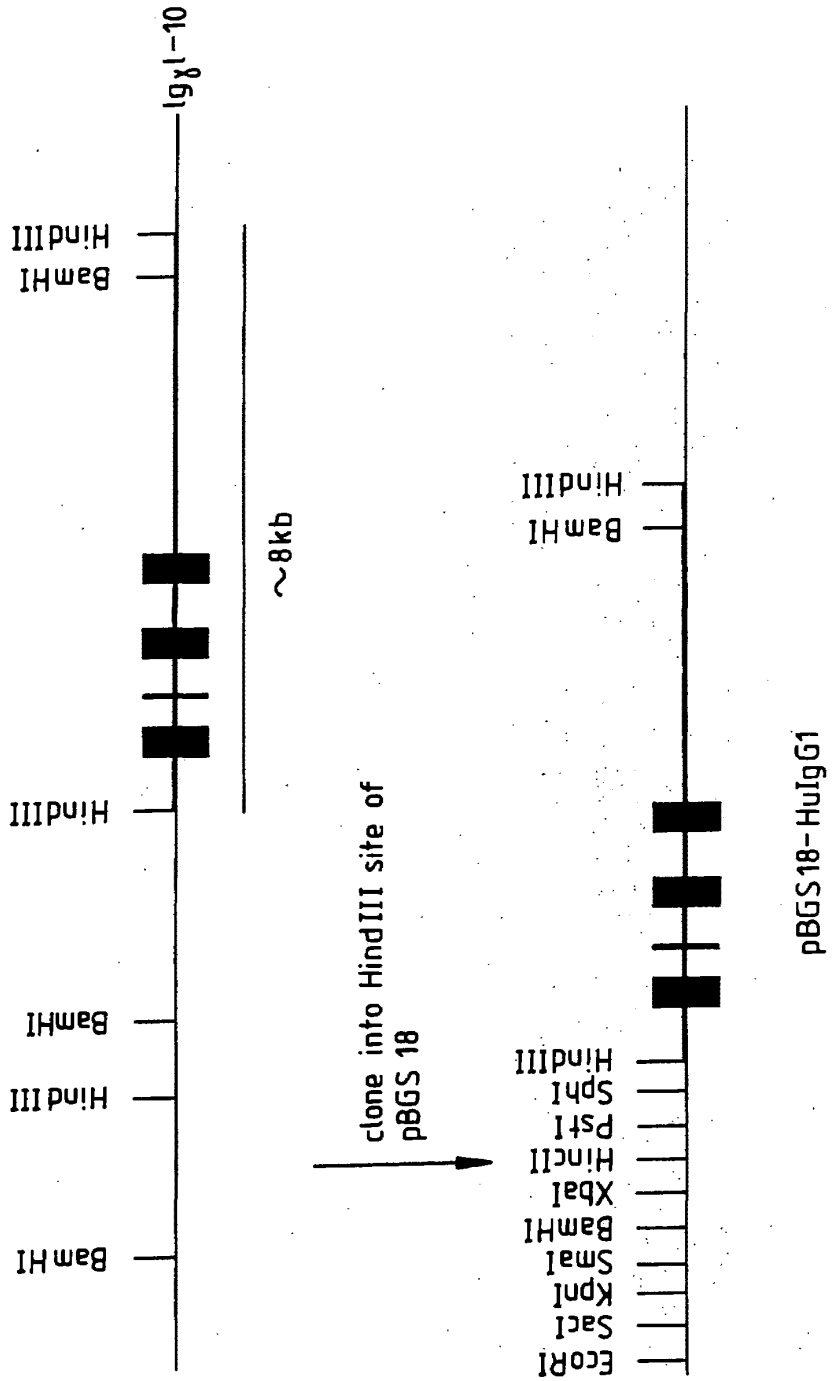
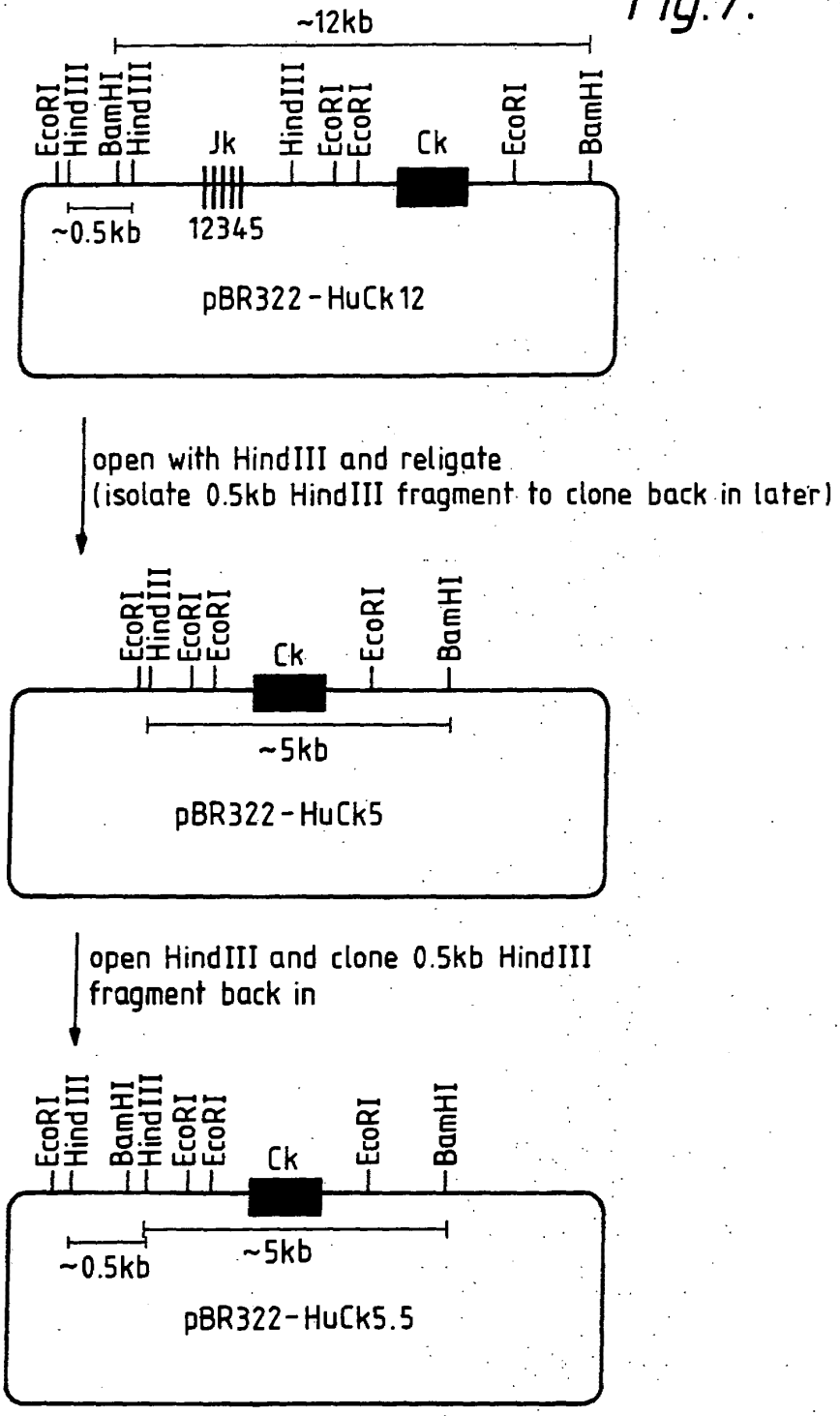


Fig. 7.



Subclone HuCk containing BamHI fragment in pBGS18 - BamHI gives: pBGS18 - HuCk

Fig. 8.

Oligonucleotides used for cloning variable region genes

I : mouse constant gamma1 primer

5' GAT AGA CAG ATG GGG GTG TCG TTT 3'

II : mouse constant kappa primer

5' AGA TGG ATA CAG TTG GTG CAG CAT 3'

Fig. 9.

Oligonucleotides used for CDR grafting

III : VHPLAP-CDR1

5' CTG TCT CAC CCA GCT TAC ACC ATA ACT *GGT* GAA GGT *GAA* GCC

AGA CAC GGT 3'.

IV : VHPLAP-CDR2

5' CAT TGT CAC TCT GGA TAT GAG AGC TGA ATG ATA ATT TGT GCT

CCC GTC TTC CCA TAT TAC TCC AAT CCA CTC 3'

V : VHPLAP-CDR3

5' GCC TTG ACC CCA GTA TTC CAT AGC CCC CAC GTA GCT GCT ACC

GTA GTG GGG TCT TGC ACA ATA 3'

Fig.9(cont.)

VI : VkPLAP-CDR1

5' CTG CTG GTA CCA TGC TAC ATA ACT GTA AAT ATT TTC ACT TGC

TCG ACA GGT GAT GGT 3'

VII : VkPLAP-CDR2

5' GCT TGG CAC ACC CTC TGC TAA GGA TTT TGC ATT GTA GAT CAG

CAG 3'

VIII : VkPLAP-CDR3

5' CCC TTG GCC GAA CGT CCA CGG ACT AAC ATA ATG ATG TTG

GCA GTA GTA GGT 3'

Fig. 10. Hu2VHPLAP

```
                    5                  10                  15                  20
CAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA CCT AGC CAG ACC CTG AGC CTG      60
Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln Thr Leu Ser Leu

                    25                  30      CDR 1       35                  40
ACC TGC ACC GTG TCT GGC TTC ACC TTC ACC AGT TAT GGT GTA AGC TGG GTG AGA CAG CCA     120
Thr Cys Thr Val Ser Gly Phe Thr Phe Thr Ser Tyr Gly Val Ser Trp Val Arg Gln Pro

                    45                  50                  55      CDR 2       60
CCT GGA CGA GGT CTT GAG TGG ATT GGA GTA ATA TGG GAA GAC GGG AGC ACA AAT TAT CAT     180
Pro Gly Arg Gly Leu Glu Trp Ile Gly Val Ile Trp Glu Asp Gly Ser Thr Asn Tyr His

                    65                  70                  75                  80
TCA GCT CTC ATA TCC AGA GTG ACA ATG CTG GTA GAC ACC AGC AAG AAC CAG TTC AGC CTG     240
Ser Ala Leu Ile Ser Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln Phe Ser Leu

     82  A   B   C  83      85                  90                  95      CDR 3
AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC TAT TAT TGT GCA AGA CCC CAC TAC     300
Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala Arg Pro His Tyr

         100  A   B   C   D   E  101                 105                 110
GGT AGC AGC TAC GTG GGG GCT ATG GAA TAC TGG GGT CAA GGC AGC CTC GTC ACA GTC TCC     360
Gly Ser Ser Tyr Val Gly Ala Met Glu Tyr Trp Gly Gln Gly Ser Leu Val Thr Val Ser

TCA                                                                               363
Ser
```

Fig.11.

HuVkPLAP

```
                    5                  10                  15                  20
GAC ATC CAG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT GAC AGA GTG ACC     60
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr

                   25       CDR 1      30                  35                  40
ATC ACC TGT CGA GCA AGT GAA AAT ATT TAC AGT TAT GTA GCA TGG TAC CAG CAG AAG CCA    120
Ile Thr Cys Arg Ala Ser Glu Asn Ile Tyr Ser Tyr Val Ala Trp Tyr Gln Gln Lys Pro

                   45                  50      CDR 2       55                  60
GGT AAG GCT CCA AAG CTG CTG ATC TAC AAT GCA AAA TCC TTA GCA GAG GGT GTG CCA AGC    180
Gly Lys Ala Pro Lys Leu Leu Ile Tyr Asn Ala Lys Ser Leu Ala Glu Gly Val Pro Ser

                   65                  70                  75                  80
AGA TTC AGC GGT AGC GGT AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC CAG CCA    240
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro

                   85                  90      CDR 3       95                  100
GAG GAC ATC GCC ACC TAC TAC TGC CAA CAT CAT TAT GTT AGT CCG TGG ACG TTC GGC CAA    300
Glu Asp Ile Ala Thr Tyr Tyr Cys Gln His His Tyr Val Ser Pro Trp Thr Phe Gly Gln

                   105
GGG ACC AAG GTG GAA ATC AAA CGT                                                   324
Gly Thr Lys Val Glu Ile Lys Arg
```

Fig. 12.

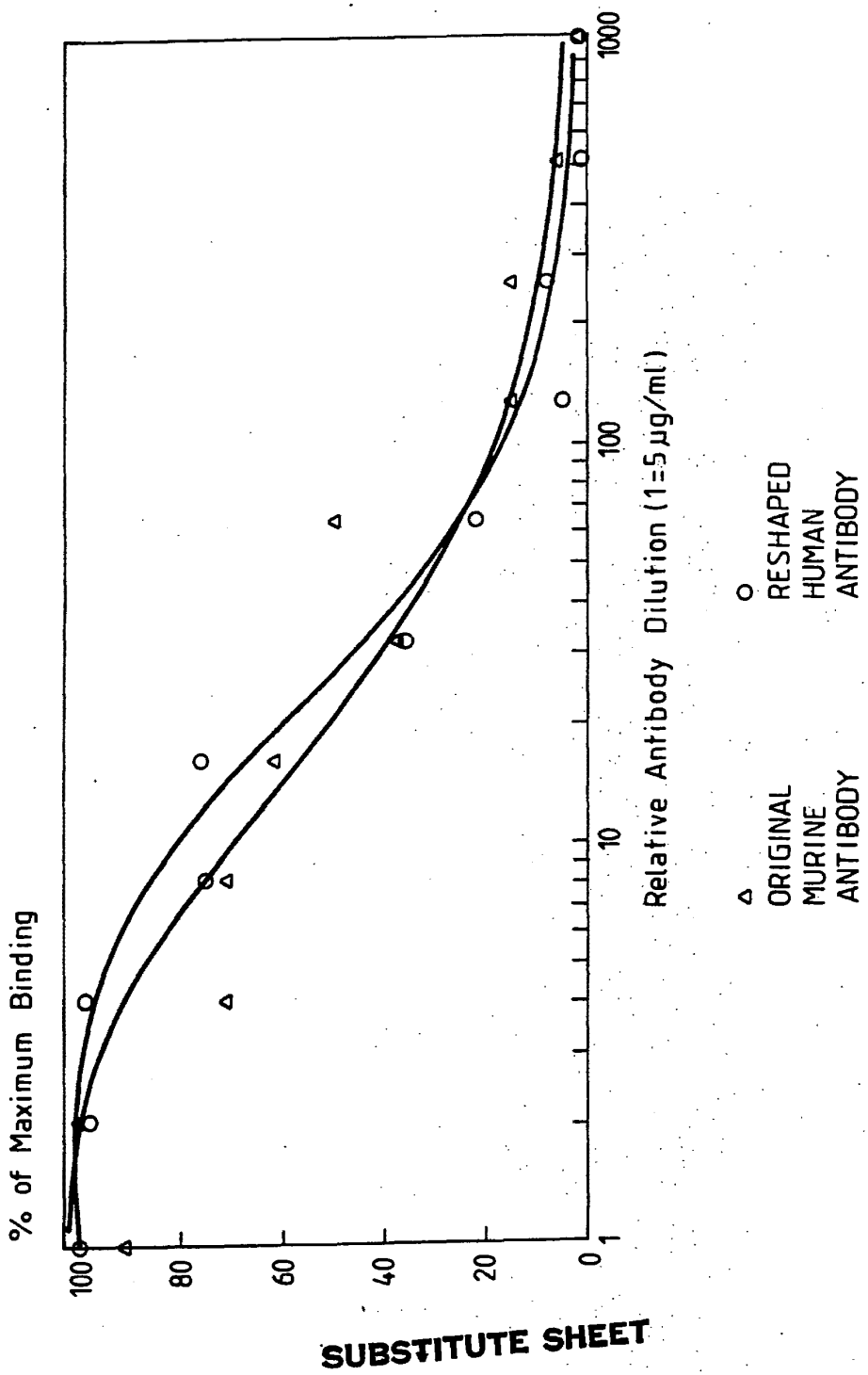
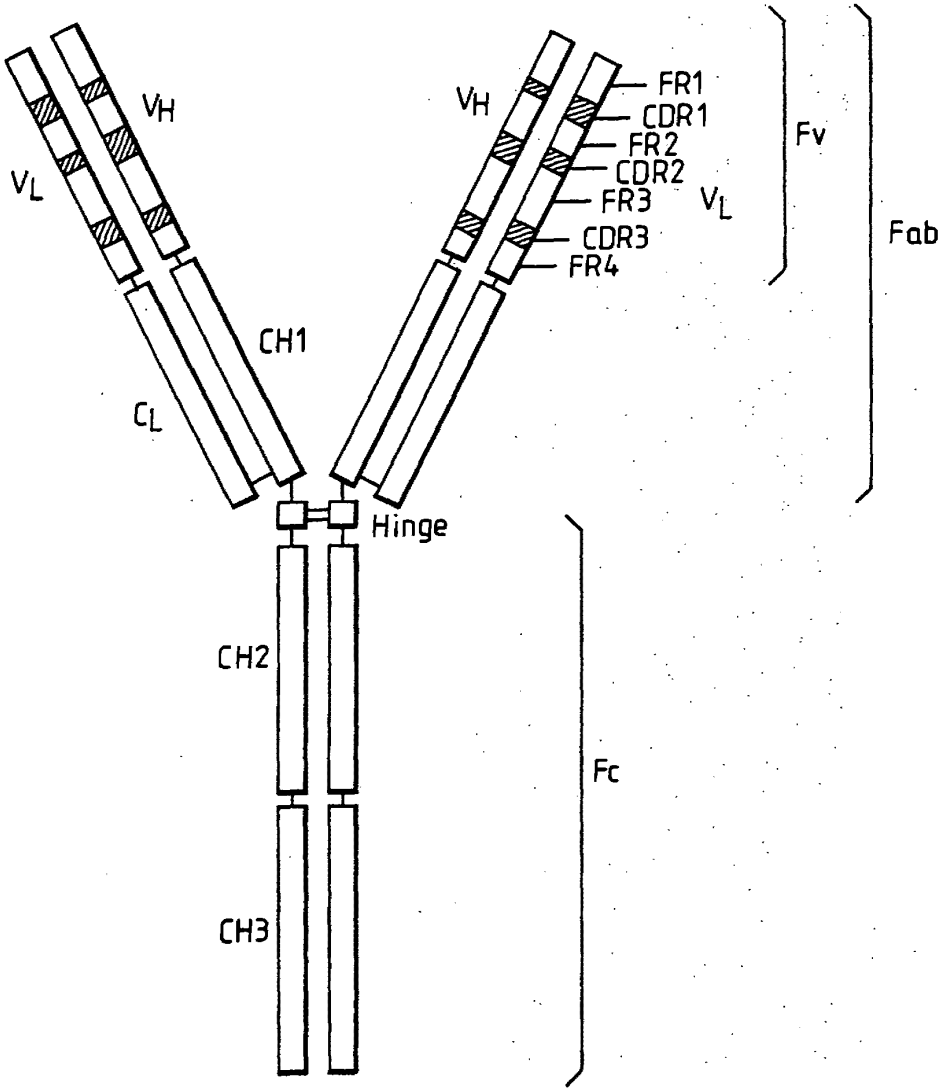


Fig.13.



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01755

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC C 12 P 21/08,

IPC[5]: C 12 N 15/13, C 12 N 5/10, A 61 K 39/395, G 01 N 33/574

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, C 12 P, C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	European Journal of Biochemistry, volume 176, no. 2, 1988, P. De Waele et al.: "Expression in non-lymphoid cells of mouse recombinant immunoglobulin directed against the tumour marker human placental alkaline phosphatase", pages 287-295 see the whole article	1,2,11, 28-30
Y	---	3-10,12-27
Y	EP, A, 0239400 (G.P. WINTER) 30 September 1987 see the whole document cited in the application	3-10,12-27
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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th March 1991	10 APR 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS D.S. KOWALCZYK

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 31, because they relate to subject matter not required to be searched by this Authority, namely:

Pls. see Rule 39.1 (iv) - PCT:

Method for treatment of the human or animal body by therapy or surgery , as well as diagnostic method.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

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Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9001755 SA 42041

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 03/04/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0239400	30-09-87	GB-A,B 2188638	07-10-87
		JP-A- 62296890	24-12-87

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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9110549.4	15 May 1991 (15.05.91)	GB

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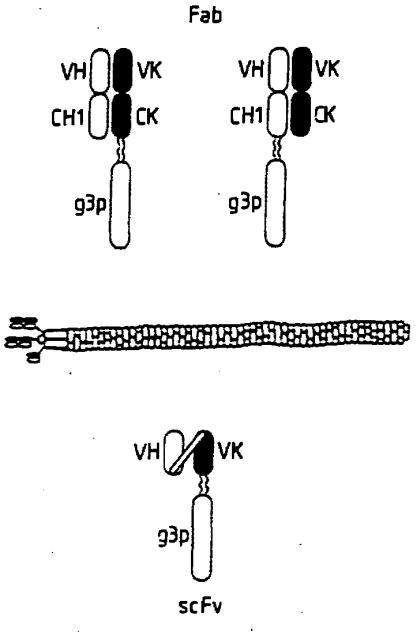
With international search report.

(54) Title: METHODS FOR PRODUCING MEMBERS OF SPECIFIC BINDING PAIRS

(57) Abstract

A member of a specific binding pair (sbp) is identified by expressing DNA encoding a genetically diverse population of such sbp members in recombinant host cells in which the sbp members are displayed in functional form at the surface of a secreted recombinant genetic display package (rgdp) containing DNA encoding the sbp member or a polypeptide component thereof, by virtue of the sbp member or a polypeptide component thereof being expressed as a fusion with a capsid component of the rgdp. The displayed sbps may be selected by affinity with a complementary sbp member, and the DNA recovered from selected rgdps for expression of the selected sbp members. Antibody sbp members may be thus obtained, with the different chains thereof expressed, one fused to the capsid component and the other in free form for association with the fusion partner polypeptide. A phagemid may be used as an expression vector, with said capsid fusion helping to package the phagemid DNA. Using this method libraries of DNA encoding respective chains of such multimeric sbp members may be combined, thereby obtaining a much greater genetic diversity in the sbp members than could easily be obtained by conventional methods.



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+ DESIGNATIONS OF "SU"

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METHODS FOR PRODUCING MEMBERS OF SPECIFIC BINDING PAIRS

The present invention relates to methods for producing members of specific binding pairs. The present invention also relates to the biological binding molecules produced by these methods.

Owing to their high specificity for a given antigen, the advent of monoclonal antibodies (Kohler, G. and Milstein C; 1975 Nature 256: 495) represented a significant technical break-through with important consequences both scientifically and commercially.

Monoclonal antibodies are traditionally made by establishing an immortal mammalian cell line which is derived from a single immunoglobulin producing cell secreting one form of a biologically functional antibody molecule with a particular specificity. Because the antibody-secreting mammalian cell line is immortal, the characteristics of the antibody are reproducible from batch to batch. The key properties of monoclonal antibodies are their specificity for a particular antigen and the reproducibility with which they can be manufactured.

Structurally, the simplest antibody (IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulphide bonds (see figure 1). The light chains exist in two distinct forms called kappa (K) and lambda (λ). Each chain has a constant region (C) and a variable region (V). Each chain is organized into a series of domains. The light chains have two domains, corresponding to the C region and the other to the V region. The heavy chains have four domains, one corresponding to the V region and three domains (1,2 and 3) in the C region. The antibody has two arms (each arm being a Fab region), each of which has a VL and a VH region associated with each other. It is this pair of V regions (VL and VH) that differ from one antibody to another (owing to amino acid sequence variations), and which together are responsible for recognising the antigen and providing an antigen binding site (ABS). In even more detail, each V region is made up from three complementarity determining regions (CDR) separated by four framework regions (FR). The CDR's are the most variable part of the variable regions, and they perform the critical antigen binding function. The CDR regions are derived from many potential germ line sequences via a complex process involving recombination, mutation and selection.

It has been shown that the function of binding antigens can be performed by fragments of a whole antibody. Example binding fragments are (i) the Fab fragment consisting of the VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989) which consists of a VH domain; (v) isolated CDR

regions; and (vi) $F(ab')_2$ fragments, a bivalent fragment comprising two Fab fragments linked by a disulphide bridge at the hinge region.

Although the two domains of the Fv fragment are coded for by separate genes, it has proved possible to make a synthetic linker that enables them to be made as a single protein chain (known as single chain Fv (scFv); Bird, R.E. et al., Science 242, 423-426 (1988) Huston, J.S. et al., Proc. Natl. Acad. Sci., USA 85, 5879-5883 (1988)) by recombinant methods. These scFv fragments were assembled from genes from monoclonals that had been previously isolated. In this application, the applicants describe a process to assemble scFv fragments from VH and VL domains that are not part of an antibody that has been previously isolated.

Whilst monoclonal antibodies, their fragments and derivatives have been enormously advantageous, there are nevertheless a number of limitations associated with them.

Firstly, the therapeutic applications of monoclonal antibodies produced by human immortal cell lines holds great promise for the treatment of a wide range of diseases (Clinical Applications of Monoclonal Antibodies. Edited by E. S. Lennox. British Medical Bulletin 1984. Publishers Churchill Livingstone). Unfortunately, immortal antibody-producing human cell lines are very difficult to establish and they give low yields of antibody (approximately 1 µg/ml). In contrast, equivalent rodent cell lines yield high amounts of antibody (approximately 100 µg/ml). However, the repeated administration of these foreign rodent proteins to humans can lead to harmful hypersensitivity reactions. In the main therefore, these rodent-derived monoclonal antibodies have limited therapeutic use.

Secondly, a key aspect in the isolation of monoclonal antibodies is how many different clones of antibody producing cells with different specificities, can be practically established and sampled compared to how many theoretically need to be sampled in order to isolate a cell producing antibody with the desired specificity characteristics (Milstein, C., Royal Soc. Croonian Lecture, Proc. R. Soc. London B. 239; 1-16, (1990)). For example, the number of different specificities expressed at any one time by lymphocytes of the murine immune system is thought to be approximately 10^7 and this is only a small proportion of the potential repertoire of specificities. However, during the isolation of a typical antibody producing cell with a desired specificity, the investigator is only able to sample 10^3 to 10^4 individual specificities. The problem is worse in the human, where one has approximately 10^{12} lymphocyte specificities, with the limitation on sampling of 10^3 or 10^4 remaining.

This problem has been alleviated to some extent in laboratory animals by the use of immunisation regimes. Thus, where one wants to produce monoclonal antibodies having a specificity against a particular epitope, an animal

is immunised with an immunogen expressing that epitope. The animal will then mount an immune response against the immunogen and there will be a proliferation of lymphocytes which have specificity against the epitope. Owing to this proliferation of lymphocytes with the desired specificity, it becomes easier to detect them in the sampling procedure. However, this approach is not successful in all cases, as a suitable immunogen may not be available. Furthermore, where one wants to produce human monoclonal antibodies (eg for therapeutic administration as previously discussed), such an approach is not practically, or ethically, feasible.

In the last few years, these problems have in part, been addressed by the application of recombinant DNA methods to the isolation and production of e.g. antibodies and fragments of antibodies with antigen binding ability, in bacteria such as E.coli.

This simple substitution of immortalised cells with bacterial cells as the 'factory', considerably simplifies procedures for preparing large amounts of binding molecules. Furthermore, a recombinant production system allows scope for producing tailor-made antibodies and fragments thereof. For example, it is possible to produce chimaeric molecules with new combinations of binding and effector functions, humanised antibodies (e.g. murine variable regions combined with human constant domains or murine-antibody CDRs grafted onto a human FR) and novel antigen-binding molecules. Furthermore, the use of polymerase chain reaction (PCR) amplification (Saiki, R.K., et al., Science 239, 487-491 (1988)) to isolate antibody producing sequences from cells (e.g. hybridomas and B cells) has great potential for speeding up the timescale under which specificities can be isolated. Amplified VH and VL genes are cloned directly into vectors for expression in bacteria or mammalian cells (Orlandi, R., et al., 1989, Proc. Natl. Acad. Sci., USA 86, 3833-3837; Ward, E.S., et al., 1989 supra; Larrick, J.W., et al., 1989, Biochem. Biophys. Res. Commun. 160, 1250-1255; Sastry, L. et al., 1989, Proc. Natl. Acad. Sci., USA., 86, 5728-5732). Soluble antibody fragments secreted from bacteria are then screened for binding activities.

However, like the production system based upon immortalised cells, the recombinant production system still suffers from the selection problems previously discussed and therefore relies on animal immunization to increase the proportion of cells with desired specificity. Furthermore, some of these techniques can exacerbate the screening problems. For example, large separate H and L chain libraries have been produced from immunized mice and combined together in a random combinatorial manner prior to screening (Huse, W.D. et al., 1989, Science 246, 1275-1281, WO90/14443; WO90/14424 and WO90/14430). Crucially however, the information held within each cell, namely the original pairing of one L chain with one H chain, is lost. This loses some, of the advantage gained by using immunization protocols in the animal. Currently, only libraries derived from single VH domains (dAbs; Ward, E.S., et al., 1989,

supra.) do not suffer this drawback. However, because not all antibody VH domains are capable of binding antigen, more have to be screened. In addition, the problem of directly screening many different specificities in prokaryotes remains to be solved.

Thus, there is a need for a screening system which ameliorates or overcomes one or more of the above or other problems. The ideal system would allow the sampling of very large numbers of specificities (eg 10^6 and higher), rapid sorting at each cloning round, and rapid transfer of the genetic material coding for the binding molecule from one stage of the production process, to the next stage.

The most attractive candidates for this type of screening, would be prokaryotic organisms (because they grow quickly, are relatively simple to manipulate and because large numbers of clones can be created) which express and display at their surface a functional binding domain eg. an antibody, receptor, enzyme etc. In the UK patent GB 2137631B methods for the co-expression in a single host cell of the variable H and L chain genes of immunoglobulins were disclosed. However, the protein was expressed intracellularly and was insoluble. Further, the protein required extensive processing to generate antibody fragments with binding activity and this generated material with only a fraction of the binding activity expected for antibody fragments at this concentration. It has already been shown that antibody fragments can be secreted through bacterial membranes with the appropriate signal peptide (Skerra, A. and Pluckthun, A. 1988 Science 240 1038-1040; Better, M et al 1988, Science 240 1041-1043) with a consequent increase in the binding activity of antibody fragments. These methods require screening of individual clones for binding activity in the same way as do mouse monoclonal antibodies.

It has not been shown however, how a functional binding domain eg an antibody, antibody fragment, receptor, enzyme etc can be held on the bacterial surface in a configuration which allows sampling of say its antigen binding properties and selection for clones with desirable properties. In large part, this is because the bacterial surface is a complex structure, and in the gram-negative organisms there is an outer wall which further complicates the position. Further, it has not been shown that eg an antibody domain will fold correctly when expressed as a fusion with a surface protein of bacteria or bacteriophage.

Bacteriophage are attractive prokaryote related organisms for this type of screening. In general, their surface is a relatively simple structure, they can be grown easily in large numbers, they are amenable to the practical handling involved in many potential mass screening programmes, and they carry genetic information for their own synthesis within a small, simple package. The difficulty has been to practically solve the problem of how to use bacteriophages in this manner. A Genex Corporation patent application number WO88/06630 has proposed that the bacteriophage lambda would be a suitable vehicle for the

expression of antibody molecules, but they do not provide a teaching which enables the general idea to be carried out. For example WO88/06630 does not demonstrate that any sequences: (a) have been expressed as a fusion with gene V; (b) have been expressed on the surface of lambda; and (c) have been expressed so that the protein retains biological activity. Furthermore there is no teaching on how to screen for suitable fusions. Also, since the lambda virions are assembled within the cell, the fusion protein would be expressed intracellularly and would be predicted to be inactive. Bass et al., in December 1990 (after the earliest priority date for the present application) describe deleting part of gene III of the filamentous bacteriophage M13 and inserting the coding sequence for human growth hormone (hGH) into the N-terminal site of the gene. The growth hormone displayed by M13 was shown to be functional. (Bass, S., et al. Proteins, Structure, Function and Genetics (1990) 8: 309-314). A functional copy of gene III was always present in addition, when this fusion was expressed. A Protein Engineering Corporation patent application WO90/02809 proposes the insertion of the coding sequence for bovine pancreatic trypsin inhibitor (BPTI) into gene VIII of M13. However, the proposal was not shown to be operative. For example, there is no demonstration of the expression of BPTI sequences as fusions with protein VIII and display on the surface of M13. Furthermore this document teaches that when a fusion is made with gene III, it is necessary to use a second synthetic copy of gene III, so that some unaltered gene III protein will be present. The embodiments of the present application do not do this. In embodiments where phagemid is rescued with M13K07 gene III deletion phage, there is no unaltered gene III present.

WO90/02809 also teaches that phagemids that do not contain the full genome of M13 and require rescue by coinfection with helper phage are not suitable for these purposes because coinfection could lead to recombination.

In all embodiments where the present applicants have used phagemids, they have used a helper phage and the only sequences derived from filamentous bacteriophage in the phagemids are the origin of replication and gene III sequences.

WO90/02809 also teaches that their process needed information such as nucleotide sequence of the starting molecule and its three-dimensioned structure. The use of a pre-existing repertoire of binding molecules to select for a binding member, such as is disclosed herein, for example using an immunoglobulin gene repertoire of animals, was not disclosed. Further, they dc not discuss favouring variegation of their binding molecules in natural blocks of variation such as CDRs of immunoglobulins, in order to favour generation of improved molecules and prevent unfavourable variations. WO90/02809 also specifically excluded the application of their process to the production of scFv molecules.

In each of the above discussed patents (WO88/06630 and

WO90/02809), the protein proposed for display is a single polypeptide chain. There is no disclosure of a method for the display of a dimeric molecule by expression of one monomer as a fusion with a capsid protein and the other protein in a free form.

Another disclosure published in May 1991 (after the earliest priority date for the present application) describes the insertion into gene VIII of M13, the coding sequences for one of the two chains of the Fab portion of an antibody with co-expression of the other from a plasmid. The two chains were demonstrated as being expressed as a functional Fab fragment on the surface of the phage (Kang A.S. et al., (1991) Proc. Natl. Acad. Sci, USA, 88 p4363-4366). No disclosure was made of the site of insertion into gene VIII and the assay for pAb binding activity by ELISA used a reagent specific for antibody L chain rather than for phage. A further disclosure published in March 1991 (after the earliest priority date for the present application) describes the insertion of a fragment of the AIDS virus protein gag into the N-terminal portion of gene III of the bacteriophage fd. The expression of the gag protein fragment was detected by immunological methods, but it was not shown whether or not the protein was expressed in a functional form (Tsunetsugu-Yokota Y et al. (1991) Gene 99 p261-265).

The problem of how to use bacteriophages in this way is in fact a difficult one. The protein must be inserted into the phage in such a way that the integrity of the phage coat is not undermined, and the protein itself should be functional retaining its biological activity with respect to antigen binding. Thus, where the protein of choice is an antibody, it should fold efficiently and correctly and be presented for antigen binding. Solving the problem for antibody molecules and fragments would also provide a general method for any biomolecule which is a member of a specific binding pair e.g. receptor molecules and enzymes.

Surprisingly, the applicants have been able to construct a bacteriophage that expresses and displays at its surface a large biologically functional binding molecule (eg antibody fragments, and enzymes and receptors) and which remains intact and infectious. The applicants have called the structure which comprises a virus particle and a binding molecule displayed at the viral surface a 'package'. Where the binding molecule is an antibody, an antibody derivative or fragment, or a domain that is homologous to an immunoglobulin domain, the applicants call the package a 'phage antibody' (pAb). However, except where the context demands otherwise, where the term phage antibody is used generally, it should also be interpreted as referring to any package comprising a virus particle and a biologically functional binding molecule displayed at the viral surface.

pAbs have a range of applications in selecting antibody genes encoding antigen binding activities. For example, pAbs could be used for the cloning and rescue of hybridomas

(Orlandi, R., et al (1989) PNAS 86 p3833-3837), and in the screening of large combinatorial libraries (such as found in Huse, W.D. et al., 1989, Science 246, 1275-1281). In particular, rounds of selection using pAbs may help in rescuing the higher affinity antibodies from the latter libraries. It may be preferable to screen small libraries derived from antigen-selected cells (Casali, P., et al., (1986) Science 234 p476-479) to rescue the original VH/VL pairs comprising the Fv region of an antibody. The use of pAbs may also allow the construction of entirely synthetic antibodies. Furthermore, antibodies may be made which have some synthetic sequences e.g. CDRs, and some naturally derived sequences. For example, V-gene repertoires could be made in vitro by combining un-rearranged V genes, with D and J segments. Libraries of pAbs could then be selected by binding to antigen, hypermutated in vitro in the antigen-binding loops or V domain framework regions, and subjected to further rounds of selection and mutagenesis.

As previously discussed, separate H and L chain libraries lose the original pairing between the chains. It is difficult to make and screen a large enough library for a particularly advantageous combination of H and L chains.

For example, in a mouse there are approximately 10^7 possible H chains and 10^7 possible L chains. Therefore, there are 10^{14} possible combinations of H and L chains, and to test for anything like this number of combinations one would have to create and screen a library of about 10^{14} clones. This has not previously been a practical possibility.

The present invention provides a number of approaches which ameliorate this problem.

In a first approach, (a random combinatorial approach, see examples 20 and 21) as large a library as is practically possible is created which expresses as many of the 10^{14} potential combinations as possible. However, by virtue of the expression of the H and L chains on the surface of the phage, it is reasonably practicable to select the desired combination, from all the generated combinations by affinity techniques (see later for description of selection formats).

In a second approach (called a dual combinatorial approach by the present applicants, see example 26), a large library is created from two smaller libraries for selection of the desired combination. This ameliorates the problems still further. The approach involves the creation of: (i) a first library of say 10^7 e.g. H chains which are displayed on a bacteriophage (as a fusion with the protein encoded by gene III) which is resistant to e.g. tetracycline; and (ii) a second library of say 10^7 e.g. L chains in which the coding sequences for these light chains are within a plasmid vector containing an origin of replication for a bacteriophage (a phagemid) which is resistant to e.g. ampicillin (i.e. a different antibiotic) and are expressed in the periplasmic space of a host bacterium. The first library is then used to infect the bacteria containing the second library to provide 10^{14} combinations of H and L

chains on the surface of the resulting phage in the bacterial supernatant.

The advantage of this approach is that two separate libraries of eg 10^7 are created in order to produce 10^{14} combinations. Creating a 10^7 library is a practical possibility.

The 10^{14} combinations are then subjected to selection (see later for description of selection formats) as disclosed by the present application. This selection will then produce a population of phages displaying a particular combination of H and L chains having the desired specificity. The phages selected however, will only contain DNA encoding one partner of the paired H and L chains (deriving from either the phage or phagemid). The sample eluate containing the population is then divided into two portions. A first portion is grown on e.g. tetracycline plates to select those bacteriophage containing DNA encoding H chains which are involved in the desired antigen binding. A second portion is grown on e.g. ampicillin plates to select those bacteriophage containing phagemid DNA encoding L chains which are involved in the desired antigen binding. A set of colonies from individually isolated clones e.g. from the tetracycline plates are then used to infect specific colonies e.g. from the ampicillin plates. This results in bacteriophage expressing specific combinations of H and L chains which can then be assayed for antigen binding.

In a third approach (called a hierarchical dual combinational approach by the present applicants), an individual colony from either the H or L chain clone selected by growth on the antibiotic plates, is used to infect a complete library of clones encoding the other chain (H or L). Selection is as described above. This favours isolation of the most favourable combination.

In a fourth approach (called a hierarchrical approach by the present applicants, see examples 22 and 46) both chains are cloned into the same vector. However, one of the chains which is already known to have desirable properties is kept fixed. A library of the complementary chain is inserted into the same vector. Suitable partners for the fixed chain are selected following display on the surface of bacteriophage.

In a fifth approach (see example 48), to improve the chances of recovering original pairs, the complexity of the combinatorial libraries can be reduced by using small B populations of B-lymphocytes selected for binding to a desired antigen. The cells provide e.g. mRNA or DNA, for preparing libraries of antibody genes for display on phage. This technique can be used in combination with the above mentioned four approaches for selection of antibody specificities.

Phagemids have been mentioned above. The applicants have realised and demonstrated that in many cases phagemids will be preferred to phage for cloning antibodies because it is easier to use them to generate more comprehensive

libraries of the immune repertoire. This is because the phagemid DNA is approximately 100 times more efficient than bacteriophage DNA in transforming bacteria (see example 19). Also, the use of phagemids gives the ability to vary the number of gene III binding moecule fusion proteins displayed on the surface of the bacteriophage (see example 17). For example, in a system comprising a bacterial cell containing a phagemid encoding a gene III fusion protein and infected with a helper phage, induction of expression of the gene III fusion protein to different extents, will determine the number of gene III fusion proteins present in the space defined between the inner and outer bacterial membranes following superinfection. This will determine the ratio of gene III fusion protein to native gene III protein displayed by the assembled phage.

Expressing a single fusion protein per virion may aid selection of antibody specificities on the basis of affinity by avoiding the 'avidity' effect where a phage expressing two copies of a low affinity antibody would have the same apparent affinity as a phage expressing one copy of a higher affinity antibody. In some cases however, it will be important to display all the gene III molecules derived by superinfection of cells containing phagemids to have fusions (e.g. for selecting low affinity binding molecules or improving sensitivity on ELISA). One way to do this is to superinfect with a bacteriophage which contains a defective gene III. The applicants have therefore developed and used a phage which is deleted in gene III. This is completely novel.

The demonstration that a functional antigen-binding domain can be displayed on the surface of phage, has implications beyond the construction of novel antibodies. For example, if other protein domains can be displayed at the surface of a phage, phage vectors could be used to clone and select genes by the binding properties of the displayed protein. Furthermore, variants of proteins, including epitope libraries built into the surface of the protein, could be made and readily selected for binding activities. In effect, other protein architectures might serve as "nouvelle" antibodies.

The technique provides the possibility of building antibodies from first principles, taking advantage of the structural framework on which the antigen binding loops fold. In general, these loops have a limited number of conformations which generate a variety of binding sites by alternative loop combinations and by diverse side chains. Recent successes in modelling antigen binding sites augurs well for de novo design. In any case, a high resolution structure of the antigen is needed. However, the approach is attractive for making e.g. catalytic antibodies, particularly for small substrates. Here side chains or binding sites for prosthetic groups might be introduced, not only to bind selectively to the transition state of the substrate, but also to participate directly in bond making and breaking. The only question is whether the antibody

architecture, specialised for binding, is the best starting point for building catalysts. Genuine enzyme architectures, such as the triose phosphate isomerase (TIM) barrel, might be more suitable. Like antibodies, TIM enzymes also have a framework structure (a barrel of β-strands and α-helices) and loops to bind substrate. Many enzymes with a diversity of catalytic properties are based on this architecture and the loops might be manipulated independently on the frameworks for design of new catalytic and binding properties. The phage selection system as provided by the present disclosure can be used to select for antigen binding activities and the CDR loops thus selected, used on either an antibody framework or a TIM barrel framework. Loops placed on a e.g. a TIM barrel framework could be further modified by mutagenesis and subjected to further selection. Thus, there is no need to select for high affinity binding activities in a single step. The strategy of the immune system, in which low affinity evolves to high affinity seems more realistic and can be mimicked using this invention.

One class of molecules that could be useful in this type of application are receptors. For example, a specific receptor could be displayed on the surface of the phage such that it would bind its ligand. The receptor could then be modified by, for example, in vitro mutagenesis and variants having higher binding affinity for the ligand selected. The selection may be carried out according to one or more of the formats described below with reference to figure 2 (which refers particularly to pAbs) in which the pAb antibody is replaced with a phage receptor and the antigen with a ligand 1.

Alternatively, the phage-receptor could be used as the basis of a rapid screening system for the binding of ligands, altered ligands, or potential drug candidates. The advantages of this system namely of simple cloning, convenient expression, standard reagents and easy handling makes the drug screening application particularly attractive. In the context of this discussion, receptor means a molecule that binds a specific, or group of specific, ligand(s). The natural receptor could be expressed on the surface of a population of cells, or it could be the extracellular domain of such a molecule (whether such a form exists naturally or not), or a soluble molecule performing a natural binding function in the plasma, or within a cell or organ.

Another possibility, is the display of an enzyme molecule or active site of an enzyme molecule on the surface of a phage (see examples 11,12,30,31,32 and 36). Once the phage enzyme is expressed, it can be selected by affinity chromatography, for instance on columns derivatized with transition state analogues. If an enzyme with a different or modified specificity is desired, it may be possible to mutate an enzyme displayed as a fusion on bacteriophage and then select on a column derivatised with an analogue selected to have a higher affinity for an enzyme with the desired modified specificity.

Although throughout this application, the applicants discuss the possibility of screening for higher affinity variants of pAbs, they recognise that in some applications, for example low affinity chromatography (Ohlson, S. et al Anal. Biochem. 169, p204-208 (1988)), it may be desirable to isolate lower affinity variants.

Examples 21 and 23 show that the present invention provides a way of producing antibodies with low affinities (as seen in the primary immune response or in unimmunised animals). This is made possible by displaying multiple copies of the antibody on the phage surface in association with gene III protein. Thus, pAbs allow genes for these antibodies to be isolated and if necessary, mutated to provide improved antibodies.

pAbs also allow the selection of antibodies for improved stability. It has been noted for many antibodies, that yield and stability are improved when the antibodies are expressed at 30°C rather than 37°C. If pAbs are displayed at 37°C, only those which are stable will be available for affinity selection. When antibodies are to be used in vivo for therapeutic or diagnostic purposes, increased stability would extend the half-life of antibodies in circulation.

Although stability is important for all antibodies and antibody domains selected using phage, it is particularly important for the selection of Fv fragments which are formed by the non-covalent association of VH and VL fragments. Fv fragments have a tendency to dissociate and have a much reduced half-life in circulation compared to whole antibodies. Fv fragments are displayed on the surface of phage, by the association of one chain expressed as a gene III protein fusion with the complementary chain expressed as a soluble fragment. If pairs of chains have a high tendency to dissociate, they will be much less likely to be selected as pAbs. Therefore, the population will be enriched for pairs which do associate stably. Although dissociation is less of a problem with Fab fragments, selection would also occur for Fab fragments which associate stably. pAbs allow selection for stability to protease attack, only those pAbs that are not cleaved by proteases will be capable of binding their ligand and therefore populations of phage will be enriched for those displaying stable antibody domains.

The technique of displaying binding molecules on the phage surface can also be used as a primary cloning system. For example, a cDNA library can be constructed and inserted into the bacteriophage and this phage library screened for the ability to bind a ligand. The ligand/binding molecule combination could include any pair of molecules with an ability to specifically bind to

one another e.g. receptor/ligand, enzyme/substrate (or analogue), nucleic acid binding protein/nucleic acid etc. If one member of the complementary pair is available, this may be a preferred way of isolating a clone for the other member of the pair.

It will often be necessary to increase the diversity of a population of genes cloned for the display of their proteins on phage or to mutate an individual nucleotide sequence. Although in vitro or in vivo mutagenesis techniques could be used for either purpose, a particularly suitable method would be to use mutator strains. A mutator strain is a strain which contains a genetic defect which causes DNA replicated within it to be mutated with respect to its parent DNA. Hence if a population of genes as gene III fusions is introduced into these strains it will be further diversified and can then be transferred to a non-mutator strain, if desired, for display and selection. Example 38 covers the use of mutator strains with phage antibodies (an example of in vitro mutagenesis and selection of phage antibodies is given in example 45).

Targeted gene transfer

A useful and novel set of applications makes use of the binding protein on the phage to target the phage genome to a particular cell or group of cells. For example, a pAb specific for a cell surface molecule could be used to bind to the target cell via the surface molecule. The phage could then be internalised, either through the action of the receptor itself or as the result of another event (e.g. an electrical discharge such as in the technique of electroporation). The phage genome would then be expressed if the relevant control signals (for transcription and translation and possibly replication) were present. This would be particularly useful if the phage genome contained a sequence whose expression was desired in the target cell (along with the appropriate expression control sequences). A useful sequence might confer antibiotic resistance to the recipient cell or label the cell by the expression of its product (e.g. if the sequence expressed a detectable gene product such as a luciferase, see White, M, et al, Techniques 2(4), p194-201 (1990)), or confer a particular property on the target cell (e.g. if the target cell was a tumour cell and the new sequence directed the expression of a tumour suppressing gene), or express an antisense construct designed to turn off a gene or set of genes in the target cell, or a gene or gene product designed to be toxic to the target cell.

Alternatively, the sequence whose expression is desired in the target cell can be encoded on a phagemid. The phagemid DNA may then be incorporated into a phage displaying an antibody specific for a cell surface

receptor. For example, incorporation may be by superinfection of bacteria containing the phagemid, with a helper phage whose genome encodes the antibody fragment specific for the target cell. The package is then used to direct the phagemid to the target cell.

This technique of "targeted gene transfer" has a number of uses in research and also in therapy and diagnostics. For example, gene therapy often aims to target the replacement gene to a specific cell type that is deficient in its activity. Targetting pAbs provide a means of achieving this.

In diagnostics, phage specific for particular bacteria or groups of bacteria have been used to target marker genes, e.g. luciferase, to the bacterial host (sec, for example, Ulitzer, S., and Kuhn, J., EPA 85303913.9). If the host range of the phage is appropriate, only those bacteria that are being tested for, will be infected by the phage, express the luciferase gene and be detected by the light they emit. This system has been used to detect the presence of Salmonella. One major problem with this approach is the initial isolation of a bacteriophage with the correct host range and then the cloning of a luciferase gene cassette into that phage, such that it is functional. The pAb system allows the luciferase cassette to be cloned into a well characterised system (filamentous phage) and allows simple selection of an appropriate host range, by modifying the antibody (or other binding molecule) specificity that the pAb encodes.

The present applicants have also been able to develop novel selection systems and assay formats which depend on the unique properties of these replicable genetic display packages e.g. pAbs.

TERMINOLOGY

Much of the terminology discussed in this section has been mentioned in the text where appropriate.

Specific Binding Pair

This describes a pair of molecules (each being a member of a specific binding pair) which are naturally derived or synthetically produced. One of the pair of molecules, has an area on its surface, or a cavity which specifically binds to, and is therefore defined as complementary with a particular spatial and polar organisation of the other molecule, so that the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, receptor-ligand, enzyme-substrate, IgG-protein A.

Multimeric Member

This describes a first polypeptide which will associate with at least a second polypeptide, when the polypeptides are expressed in free form and/or on the

surface of a substrate. The substrate may be provided by a bacteriophage. Where there are two associated polypeptides, the associated polypeptide complex is a dimer, where there are three, a trimer etc. The dimer, trimer, multimer etc or the multimeric member may comprise a member of a specific binding pair.

Example multimeric members are heavy domains based on an immunoglobulin molecule, light domains based on an immunoglobulin molecule, T-cell receptor subunits.

Replicable Genetic Display Package (Rgdp)

This describes a biological particle which has genetic information providing the particle with the ability to replicate. The particle can display on its surface at least part of a polypeptide. The polypeptide can be encoded by genetic information native to the particle and/or artificially placed into the particle or an ancestor of it. The displayed polypeptide may be any member of a specific binding pair eg. heavy or light chain domains based on an immunoglobulin molecule, an enzyme or a receptor etc.

The particle may be a virus eg. a bacteriophage such as fd or M13.

Package

This describes a replicable genetic display package in which the particle is displaying a member of a specific binding pair at its surface. The package may be a bacteriophage which displays an antigen binding domain at its surface. This type of package has been called a phage antibody (pAb).

Antibody

This describes an immunoglobulin whether natural or partly or wholly synthetically produced. The term also covers any protein having a binding domain which is homologous to an immunoglobulin binding domain. These proteins can be derived from natural sources, or partly or wholly synthetically produced.

Example antibodies are the immunoglobulin isotypes and the Fab, $F(ab^1)_2$, scFv, Fv, dAb, Fd fragments.

Immunoglobulin Superfamily

This describes a family of polypeptides, the members of which have at least one domain with a structure related to that of the variable or constant domain of immunoglobulin molecules. The domain contains two β-sheets and usually a conserved disulphide bond (see A.F. Williams and A.N. Barclay 1988 Ann. Rev Immunol. 6 381-405).

Example members of an immunoglobulin superfamily are CD4, platelet derived growth factor receptor (PDGFR), intercellular adhesion molecule. (ICAM). Except where the context otherwise dictates, reference to immunoglobulins and immunoglobulin homologs in this application includes members of the immunoglobulin

superfamily and homologs thereof.

Homologs

This term indicates polypeptides having the same or conserved residues at a corresponding position in their primary, secondary or tertiary structure. The term also extends to two or more nucleotide sequences encoding the homologous polypeptides.

Example homologous peptides are the immunoglobulin isotypes.

Functional

In relation to a sbp member displayed on the surface of a rgdp, means that the sbp member is presented in a folded form in which its specific binding domain for its complementary sbp member is the same or closely analogous to its native configuration, whereby it exhibits similar specificity with respect to the complementary sbp member. In this respect, it differs from the peptides of Smith et al, supra, which do not have a definite folded configuration and can assume a variety of configurations determined by the complementary members with which they may be contacted.

Genetically diverse population

In connection with sbp members or polypeptide components thereof, this is referring not only to diversity that can exist in the natural population of cells or organisms, but also diversity that can be created by artificial mutation in vitro or in vivo.

Mutation in vitro may for example, involve random mutagenesis using oligonucleotides having random mutations of the sequence desired to be varied. In vivo mutagenesis may for example, use mutator strains of host microorganisms to harbour the DNA (see Example 38 below).

Domain

A domain is a part of a protein that is folded within itself and independently of other parts of the same protein and independently of a complementary binding member.

Folded Unit

This is a specific combination of an α-helix and/or β-strand and/or β-turn structure. Domains and folded units contain structures that bring together amino acids that are not adjacent in the primary structure.

Free Form

This describes the state of a polypeptide which is not displayed by a replicable genetic display package.

Conditionally Defective

This describes a gene which does not express a particular polypeptide under one set of conditions, but expresses it under another set of conditions. An example, is a gene containing an amber mutation expressed in non-suppressing or suppressing hosts respectively.

Alternatively, a gene may express a protein which is

defective under one set of conditions, but not under another set. An example is a gene with a temperature sensitive mutation.

Suppressible Translational Stop Codon

This describes a codon which allows the translation of nucleotide sequences downstream of the codon under one set of conditions, but under another set of conditions translation ends at the codon. Example of suppressible translational stop codons are the amber, ochre and opal codons.

Mutator Strain

This is a host cell which has a genetic defect which causes DNA replicated within it to be mutated with respect to its parent DNA. Example mutator strains are NR9046mutD5 and NR9046 mut T1 (see Example 38).

Helper Phage

This is a phage which is used to infect cells containing a defective phage genome and which functions to complement the defect. The defective phage genome can be a phagemid or a phage with some function encoding gene sequences removed. Examples of helper phages are M13K07, M13K07 gene III no. 3; and phage displaying or encoding a binding molecule fused to a capsid protein.

Vector

This is a DNA molecule, capable of replication in a host organism, into which a gene is inserted to construct a recombinant DNA molecule.

Phage Vector

This is a vector derived by modification of a phage genome, containing an origin of replication for a bacteriophage, but not one for a plasmid.

Phagemid Vector

This is a vector derived by modification of a plasmid genome, containing an origin of replication for a bacteriophage as well as the plasmid origin of replication.

Secreted

This describes a rgdp or molecule that associates with the member of a sbp displayed on the rgdp, in which the sbp member and/or the molecule, have been folded and the package assembled externally to the cellular cytosol.

Repertoire of Rearranged Immunoglobulin Genes

A collection of naturally occurring nucleotides eg DNA sequences which encoded expressed immunoglobulin genes in an animal. The sequences are generated by the in vivo rearrangement of eg V, D and J segments for H chains and eg the V and J segments for L chains. Alternatively the sequences may be generated from a cell line immunised in vitro and in which the rearrangement in response to immunisation occurs intracellularly.

Library

A collection of nucleotide eg DNA, sequences within

clones.

Repertoire of Artificially Rearranged Immunoglobulin Genes

A collection of nucleotide eg DNA, sequences derived wholly or partly from a source other than the rearranged immunoglobulin sequences from an animal. This may include for example, DNA sequences encoding VH domains by combining unrearranged V segments with D and J segments and DNA sequences encoding VL domains by combining V and J segments.

Part or all of the DNA sequences may be derived by oligonucleotide synthesis.

Secretory Leader Peptide

This is a sequence of amino acids joined to the N-terminal end of a polypeptide and which directs movement of the polypeptide out of the cytosol.

Eluant

This is a solution used to breakdown the linkage between two molecules. The linkage can be a non-covalent or covalent bond(s). The two molecules can be members of a sbp.

Derivative

This is a substance which derived from a polypeptide which is encoded by the DNA within a selected rgdp. The derivative polypeptide may differ from the encoded polypeptide by the addition, deletion, substitution or insertion of amino acids, or by the linkage of other molecules to the encoded polypetide. These changes may be made at the nucleotide or protein level. For example the encoded polypeptide may be a Fab fragment which is then linked to an Fc tail from another source. Alternatively markers such as enzymes, flouresceins etc may be linked to eg Fab, scFv fragments.

The present invention provides a method for producing a replicable genetic display package or population such rgdps of which method comprises the steps of:

a) inserting a nucleotide sequence encoding a member of a specific binding pair eg. a binding molecule within a viral genome;

b) culturing the virus containing said nucleotide sequence, so that said binding molecule is expressed and displayed by the virus at its surface.

The present invention also provides a method for selecting a rgdp specific for a particular epitope which comprises producing a population of such rgdps as described above and the additional step of selecting for said binding molecule by contacting the population with said epitope so that individual rgdps with the desired specificity may bind to said epitope. The method may comprise one or more of the additional steps of: (i) separating any bound rgdps from the epitope; (ii)

recovering any separated rgdps and (iii) using the inserted nucleotide sequences from any separated rgdps in a recombinant system to produce the binding molecule separate from virus. The selection step may isolate the nucleotide sequence encoding the binding molecule of desired specificity, by virtue of said binding molecule being expressed in association with the surface of the virus in which said encoding nucleic acid is contained.

The present invention also provides a method of producing a multimeric member of a specific binding pair (sbp), which method comprises:
expressing in a recombinant host organism a first polypeptide chain of said sbp member or a genetically diverse population of said sbp member fused to a component of a secreted replicable genetic display package (rgdp) which thereby displays said polypeptide at the surface of the package, and expressing in a recombinant host organism a second polypeptide chain of said multimer and causing or allowing the polypeptide chains come together to form said multimer as part of said rgdp at least one of said polypeptide chains being expressed from nucleic acid that is capable of being packaged using said component therefor, whereby the genetic material of each said rgdp encodes a said polypeptide chain.
Both said chains may be expressed in the same host organism.

The first and second chains of said multimer may be expressed as separate chains from a single vector containing their respective nucleic acid.

At least one of said polypeptide chains may be expressed from a phage vector.

At least one of said polypeptide chains may be expressed from a phagemid vector, the method including using a helper phage, or a plasmid expressing complementing phage genes, to help package said phagemid genome, and said component of the rgdp is a capsid protein therefor. The capsid protein may be absent, defective or conditionally defective in the helper phage.

The method may comprise introducing a vector capable of expressing said first polypeptide chain, into a host organism which expresses said second polypeptide chain in free form, or introducing a vector capable of expressing said second polypeptide in free form into a host organism which expresses said first polypeptide chain.

Each of the polypeptide chain may be expressed from nucleic acid which is capable of being packaged as a rgdp using said component fusion product, whereby encoding nucleic acid for both said polypeptide chains are packaged in respective rgdps.

The nucleic acid encoding at least one of said first and second polypeptide chains may be obtained from a

library of nucleic acid including nucleic acid encoding said chain or a population of variants of said chain. Both the first and second polypeptide chains may be obtained from respective said libraries of nucleic acid.

The present invention also provides a method of producing a member of a specific binding pair (sbp), from a nucleic acid library including nucleic acid encoding said sbp member or a genetically diverse population of said type of sbp members, which method comprises:

expressing in recombinant host cells polypeptides encoded by said library nucleic acid fused to a component of a secreted replicable genetic display package (rgdp) or in free form for association with a polypeptide component of said sbp member which is expressed as a fusion to said rgdp component so that the rgdp displays said sbp member in functional form at the surface of the package, said library nucleic acid being contained within the host cells in a form that is capable of being packaged using said rgdp component, whereby the genetic material of an rgdp displaying an sbp member contains nucleic acid encoding said sbp member or a polypeptide component thereof.

The nucleotide sequences for the libraries may be derived from eg animal spleen cells or peripheral blood lymphocytes. Alternatively the nucleotide sequence may be derived by the in vitro mutagenesis of an existing antibody coding sequence.

The present invention also provides a method of producing a member of a specific binding pair (sbp), which method comprises:

expressing in recombinant host cells nucleic acid encoding said sbp member or a genetically diverse population of said type of sbp member wherein the or each said sbp member or a polypeptide component thereof is expressed as a fusion with a component of a secreted replicable genetic display package (rgdp) which displays said sbp member at the surface of the package, nucleic acid encoding said sbp member or a polypeptide component thereof being contained within the host cell in a form that is capable of being packaged using said rgdp component whereby the genetic material of the rgdp displaying said sbp member encodes said sbp member or a polypeptide component thereof, said host organism being a mutator strain which introduces genetic diversity into the sbp member to produce said mixed population.

The present invention also provides a method of producing a member of a specific binding pair (sbp), which method comprises:

expressing in recombinant host cells nucleic acid

encoding said sbp member or a genetically diverse population of said type of sbp member wherein the or each said sbp member or a polypeptide component thereof is expressed as a fusion with a component of a secreted replicable genetic display package (rgdp) which displays said sbp member in functional form at the surface of the package, nucleic acid encoding said sbp member or a polypeptide component thereof being contained within the host cell in a form that is capable of being packaged using said rgdp component whereby the genetic material of the rgdp displaying an sbp member encodes said sbp member or a polypeptide component thereof, said fusions being with bacteriophage capsid protein and the rgdps being formed with said fusions in the absence of said capsid expressed in wild-type form.

The present invention also provides a method of producing a member of a specific binding pair (sbp) which method comprises:

expressing in recombinant host cells nucleic acid encoding said sbp member or a genetically diverse population of said type of sbp member or a polypeptide component thereof fused to a component of a secreted replicable genetic display package (rgdp) which displays said sbp member in functional form at the surface of the package, nucleic acid encoding said sbp member or a polypeptide component thereof being contained within the host cell in a form that is capable of being packaged using said rgdp component whereby the genetic material of the rgdp displaying an sbp member or a polypeptide component thereof encodes said sbp member or a polypeptide component thereof, said sbp member or polypeptide component thereof being expressed from a phagemid as a capsid fusion, and a helper phage, or a plasmid expressing complementing phage genes, is used along with said capsid fusions to package the phagemid nucleic acid.

The library or genetically diverse population may be obtained from:

(i) the repertoire of rearranged immunoglobulin genes of an animal immunised with complementary sbp member,

(ii) the repertoire of rearranged immunoglobulin genes of an animal not immunised with complementary sbp member,

(iii) a repertoire of artificially rearranged immunoglobulin gene or genes

(iv) a repertoire of immunoglobulin homolog gene or genes; or

(v) a mixture of any of (i), (ii), (iii) and (iv).

The capsid protein may be absent, defective or

conditionally defective in the helper phage.

The host cell may be a mutator strain which introduces genetic diversity into the sbp member nucleic acid.

The sbp member may comprise a domain which is, or is homologous to, an immunoglobulin domain.

The rgdp may be a bacteriophage, the host a bacterium, and said component of the rgdp a capsid protein for the bacterophage. The phage may be a filamentous phage. The phage may be selected from the class I phages fd, M13, f1, If1, 1ke, ZJ/Z, Ff and the class II phages Xf, Pf1 and Pf3. The phage may be fd or a derivative of fd. The derivative may be tetracycline resistant. The said sbp member or polypeptide chain thereof may be expressed as a fusion with the gene III capsid protein of phage fd or its counterpart in another filamentous phage. The sbp member or polypeptide chain thereof may be inserted in the N-terminal region of the mature capsid protein downstream of a secretory leader peptide. The sequence may be inserted after amino acid +1 of the mature protein. The site for insertion may be flanked by short sequences corresponding to sequences which occur at each end of the nucleic acid to be inserted. For example where 4 the protein domain is an immunoglobulin domain, the insertion site in the phage may be flanked by nucleotide sequences which code for the first five amino acids and the last five amino acids of the Ig domain. Such flanking nucleotide sequences are shown in figure 4(2) B and C, wherein the site-flanking nucleotide sequences encode amino acid sequences QVQLQ and VTVSS which occur at either end of the VH domain, or QVQLQ and LEIKR which occur at either end of the Fv (combined VH + VL) domain. Each of these sequences flanking the insertion site may include a suitable cleavage site, as shown in Fig 4.

Alternatively, the flanking nucleotide sequences shown in figure 4(2)B and C as described above, may be used to flank the insertion site for any nucleic acid to be inserted, whether or not that nucleic acid codes an immunoglobulin.

The host may be E.coli.

Nucleic acid encoding an sbp member polypeptide may be linked downstream to a viral capsid protein through a suppressible translational stop codon.

As previously mentioned, the present invention also provides novel selection systems and assay formats. In these systems and formats, the gene sequence encoding the binding molecule (eg. the antibody) of desired specificity is separated from a general population of rgdps having a range of specifies, by the fact of its binding to a specific target (eg the antigen or epitope). Thus the rgdps formed by said expression may be selected

or screened to provide an individual sbp member or a selected mixed population of said sbp members associated in their respective rgdps with nucleic acid encoding said sbp member or a polypeptide chain thereof. The rgdps may be selected by affinity with a member complementary to said sbp member.

Any rgdps bound to said second member may be recovered by washing with an eluant. The washing conditions may be varied in order to obtain rgdps with different binding affinities for said epitope. Alternatively, to obtain eg high affinity rgdps, the complementary member (eg an epitope) may be presented to the population of rgdps (eg pAbs) already bound to a binding member in which case pAbs with a higher affinity for the epitope will displace the already bound binding member. Thus the eluant may contain a molecule which competes with said rgdp for binding to the complementary sbp member. The rgdp may be applied to said complementary sbp member in the presence of a molecule which competes with said package for binding to said complementary sbp member. Nucleic acid derived from a selected or screened rgdp may be used to express said sbp member or a fragment or derivative thereof in a recombinant host organism. Nucleic acid from one or more rgdps may be taken and used to provide encoding nucleic acid in a further said method to obtain an individual sbp member or a mixed population of sbp members, or encoding nucleic acid therefor. The expression end product may be modified to produce a derivative thereof.

The expression end product or derivative thereof may be used to prepare a therapeutic or prophylactic medicament or a diagnestic product.

The present invention also provides recombinant host cells harbouring a library of nucleic acid fragments comprising fragments encoding a genetically diverse population of a type of member of a specific binding pair (sbp), each sbp member or a polypeptide component thereof being expressed as a fusion with a component of a secretable replicable genetic display package (rgdp), so that said sbp members are displayed on the surface of the rgdps in functional form and the genetic material of the rgdps encode the associated sbp member or a polypeptide component thereof. The type of sbp members may be immunoglobulins or immunoglobulin homologs, a first polypeptide chain of which is expressed as a said fusion with a component of the rgdp and a second polypeptide chain of which is expressed in free form and associates with the fused first polypeptide chain in the rgdp.

The present invention also provides a helper phage whose genome lacks nucleic acid encoding one of its capsid proteins, or whose encoding nucleic acid therefor is conditionally defective, or which encodes said capsid

protein in defective or conditionally defective form.

The present invention also provides a bacterial host cell containing a filamentous phage genome defective for a capsid protein thereof and wherein the host cell is capable of expressing capsid protein complementing said defect such that infectious phage particles can be obtained therefrom. The complementing capsid protein may be expressed in said host from another vector contained therein. The defective capsid protein may be gene III of phage fd or its counterpart in another filamentous phage.

The present invention also provides recombinant E.coli TG1 M13KO7 gIII No. 3 (NCTC 12478).

The present invention also provides a phage antibody having the form of a replicable genetic display package displaying on its surface in functional form a member of a specific binding pair or a specific binding domain thereof.

In the above methods, the binding molecule may be an antibody, or a domain that is homologous to an immunoglobulin. The antibody and/or domain may be either naturally derived or synthetic or a combination of both. The domain may be a Fab, scFv, Fv dAb or Fd molecule. Alternatively, the binding molecule may be an enzyme or receptor or fragment, derivative or analogue of any such enzyme or receptor. Alternatively, the binding molecule may be a member of an immunoglobulin superfamily and which has a structural form based on an immunoglobulin molecule.

The present invention also provides rgdps as defined above and members of specific binding pairs eg. binding molecules such as antibodies, enzymes, receptors, fragments and derivatives thereof, obtainable by use of any of the above defined methods. The derivatives may comprise members of the specific binding pairs fused to another molecule such as an enzyme or a Fc tail.

The invention also includes kits for carrying out the methods hereof. The kits will include the necessary vectors. One such vector will typically have an origin of replication for single stranded bacteriophage and either contain the sbp member nucleic acid or have a restriction site for its insertion in the 5' end region of the mature coding sequence of a phage capsid protein, and with a secretory leader coding sequence upstream of said site which directs a fusion of the capsid protein exogenous polypeptide to the periplasmic space.

The restriction sites in the vectors are preferably those of enzymes which cut only rarely in protein coding sequences.

The kit preferably includes a phagemid vector which may have the above characteristics, or may contain, or have a site for insertion, of sbp member nucleic acid for expression of the encoded polypeptide in free form.

The kits will also contain ancillary components required for carrying out the method, the nature of such components depending of course on the particular method employed.

Useful ancillary components may comprise helper phage, PCR primers, and buffers and enzymes of various kinds.

PCR primers and associated reagents for use where the sbp members are antibodies may have the following characteristics:

(i) primers having homology to the 5' end of the sense or anti-sense strand of sequences encoding domains of antibodies; and

(ii) primers including tag sequences 5' to these homologous sequences which incorporate restriction sites to allow insertion into vectors; together with sequences to allow assembly of amplified VH and VL regions to enable expression as Fv, scFv or Fab fragments.

Buffers and enzymes are typically used to enable preparation of nucleotide sequences encoding Fv, scFv or Fab fragments derived from rearranged or unrearranged immunoglobulin genes according to the strategies described herein.

The applicants have chosen the filamentous F-specific bacteriophages as an example of the type of phage which could provide a vehicle for the display of binding molecules e.g. antibodies and antibody fragments and derivatives thereof, on their surface and facilitate subsequent selection and manipulation.

The F-specific phages (e.g. f1, fd and M13) have evolved a method of propagation which does not kill the host cell and they are used commonly as vehicles for recombinant DNA (Kornberg, A., DNA Replication, W.H. Freeman and Co., San Francisco, 1980). The single stranded DNA genome (approximately 6.4 Kb) of fd is extruded through the bacterial membrane where it sequesters capsid sub-units, to produce mature virions. These virions are 6 nm in diameter, 1μm in length and each contain approximately 2,800 molecules of the major coat protein encoded by viral gene VIII and four molecules of the adsorption molecule gene III protein (g3p) the latter is located at one end of the virion. The structure has been reviewed by Webster et al., 1978 in The Single Stranded DNA Phages, 557-569, Cold Spring Harbor Laboratory Press. The gene III product is involved in the binding of the phage to the bacterial F-pilus.

Although these phages do not kill their host during normal replication, disruption of some of their genes can lead to cell death (Kornberg, A., 1980 supra.) This places some restraint on their use. The applicants have

recognized that gene III of phage fd is an attractive possibility for the insertion of biologically active foreign sequences. There are however, other candidate sites including for example gene VIII and gene VI.

The protein itself is only a minor component of the phage coat and disruption of the gene does not lead to cell death (Smith, G. 1988, Virology 167: 156-165). Furthermore, it is possible to insert some foreign sequences (with no biological function) into various positions within this gene (Smith, G. 1985 Science 228: 1315-1317., Parmley, S.F. and Smith, G.P. Gene: 73 (1988) p. 305-318., and de la Cruz, V.F., et al., 1988, J. Biol. Chem., 263: 4318-4322). Smith et al described the display of peptides on the outer surface of phage but they did not describe the display of protein domains. Peptides can adopt a range of structures which can be different when in free solution, than when bound to, for example, an antibody, or when forming part of a protein (Stanfield, R.I. et al., (1990) Science 248, p712-719). Proteins in general have a well defined tertiary structure and perform their biological function only when adopting this structure. For example, the structure of the antibody D1.3 has been solved in the free form and when bound to antigen (Bhat, T.N. et al., (1990) Nature 347, p483-485). The gross structure of the protein is identical in each instance with only minor variations around the binding site for the antigen. Other proteins have more substantial conformation changes on binding of ligand, for instance the enzymes hexokinase and pyruvate dehydrogenase during their catalytic cycle, but they still retain their overall pattern of folding. This structural integrity is not confined to whole proteins, but is exhibited by protein domains. This leads to the concept of a folded unit which is part of a protein, often a domain, which has a well defined primary, secondary and tertiary structure and which retains the same overall folding pattern whether binding to a binding partner or not. The only gene sequence that Smith et al., described that was of sufficient size to encode a domain (a minimum of perhaps 50 amino acids) was a 335bp fragment of a β-galctrosidase corresponding to nucleotides 861-1195 in the β-galactosidase gene sequence (Parmley, S. + Smith, G.P. 1988 supra. This would encode 112 amino acids of a much larger 380 amino acid domain. Therefore, prior to the present application, no substantially complete domain or folded unit had been displayed on phage. In these cases, although the infectivity of the virion was disrupted, the inserted sequences could be detected on the phage surface by use of e.g. antibodies.

The protein encoded by gene III has several domains (Pratt, D., et al., 1969 Virology 39:42-53., Grant, R.A.,

et al., 1981, J. Biol. Chem. 256: 539-546 and Armstrong, J., et al., FEBS Lett. 135: 167-172 1981.) including: (i) a signal sequence that directs the protein to the cell membrane and which is then cleaved off; (ii) a domain that anchors the mature protein into the bacterial cell membrane (and also the phage coat); and (iii) a domain that specifically binds to the phage receptor, the F-pilus of the host bacterium. Short sequences derived from protein molecules have been inserted into two places within the mature molecule (Smith, G., 1985 supra., and Parmley, S.F. and Smith G.P., 1988 supra.). Namely, into an inter-domain region and also between amino acids 2 and 3 at the N-terminus. The insertion sites at the N-terminus were more successful in maintaining the structural integrity of the gene III protein and displaying the peptides on the surface of the phage. By use of antisera specific for the peptides, the peptides inserted into this position were shown to be on the surface of the phage. These authors were also able to purify the phage, using this property. However, the peptides expressed by the phage, did not possess measurable biological functions of their own.

Retaining the biological function of a molecule when it is expressed in a radically different context to its natural state is difficult. The demands on the structure of the molecule are heavy. In contrast, retaining the ability to be bound by specific antisera is a passive process which imposes far less rigorous demands on the structure of the molecule. For example, it is the rule rather than the exception that polyclonal antisera will recognise totally denatured, and biologically inactive, proteins on Western blots (see for example, Harlow, E. and Lane, D., Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press 1988). Therefore, the insertion of peptides into a region that allows their structure to be probed with antisera teaches only that the region allows the inserted sequences to be exposed and does not teach that the region is suitable for the insertion of large sequences with demanding structural constraints for the display of a molecule with a biological or binding function. In particular, it does not teach that domains or folded units of proteins can be displayed from sequences inserted in this region.

This experience with Western blots is a graphic practical demonstration which shows that retaining the ability to be bound by specific antisera imposes far less rigorous demands on the structure of a polypeptide, than does folding for the retention of a biological function.

Studies have been carried out, in which E.coli have been manipulated to express the protein β-adrenergic receptor as a fusion with the outer membrane protein lamB. The β-adrenergic receptor was expressed in a

functional form as determined by the presence of binding activity. However, when an equivalent antibody fusion was made with lamB, the antibody fusion was toxic to the host cell.

The applicants have investigated the possibility of inserting the gene coding sequence for biologically active antibody fragments into the gene III region of fd to express a large fusion protein. As is apparent from the previous discussion, this approach makes onerous demands on the functionality of the fusion protein. The insertion is large, encoding antibody fragments of at least 100-200 amino acids; the antibody derived domain must fold efficiently and correctly to display antigen-binding; and most of the functions of gene III must be retained. The applicants approach to the construction of the fusion molecule was designed to minimise the risk of disrupting these functions. In an embodiment of the invention, the initial vector used was fd-tet (Zacher, A.N., et al., 1980, Gene 9, 127-140) a tetracycline resistant version of fd bacteriophage that can be propagated as a plasmid that confers tetracycline resistance to the infected E.coli host. The applicants chose to insert after the signal sequence of the fd gene III protein for several reasons. In particular, the applicants chose to insert after amino acid 1 of the mature protein to retain the context for the signal peptidase cleavage. To retain the structure and function of gene III itself, the majority of the original amino acids are synthesized after the inserted immunoglobulin sequences. The inserted immunoglobulin sequences were designed to include residues from the switch region that links VH-VL to CH1-CL (Lesk, A., and Chothia, C., Nature 335, 188-190, 1988).

Surprisingly, by manipulating gene III of bacteriophage fd, the present applicants have been able to construct a bacteriophage that displays on its surface large biologically functional antibody, enzyme, and receptor molecules whilst remaining intact and infectious. Furthermore, the phages bearing antibodies of desired specificity, can be selected from a background of phages not showing this specificity.

The sequences coding for a population of antibody molecules and for insertion into the vector to give expression of antibody binding functions on the phage surface can be derived from a variety of sources. For example, immunised or non-immunised rodents or humans, and from organs such as spleen and peripheral blood lymphocytes. The coding sequences are derived from these sources by techniques familiar to those skilled in the art (Orlandi, R., et al., 1989 supra; Larrick, J.W., et al., 1989 supra; Chiang, Y.L., et al., 1989 Bio Techniques 7, p. 360-366; Ward, E.S, et al., 1989 supra;

Sastry, L., et al., 1989 supra.) or by novel linkage strategies described in examples 14, 33, 40 and 42. Novel strategies are described in examples 7, 25, 33, 39 and 40 for displaying dimeric molecules eg Fab and Fv fragments on the surface of a phage. Each individual pAb in the resulting library of pAbs will express antibodies or antibody derived fragments that are monoclonal with respect to their antigen-binding characteristics.

The disclosure made by the present applicants is important and provides a significant breakthrough in the technology relating to the production of biological binding molecules, their fragments and derivatives by the use of recombinant methods.

In standard recombinant techniques for the production of antibodies, an expression vector containing sequences coding for the antibody polypeptide chains is used to transform e.g. E.coli. The antibody polypeptides are expressed and detected by use of standard screening systems. When the screen detects an antibody polypeptide of the desired specificity, one has to return to the particular transformed E.coli expressing the desired antibody polypeptide. Furthermore, the vector containing the coding sequence for the desired antibody polypeptide then has to be isolated for use from E.coli in further processing steps.

In the present invention however, the desired antibody polypeptide when expressed, is already packaged with its gene coding sequence. This means that when the an antibody polypeptide of desired specificity is selected, there is no need to return to the original culture for isolation of that sequence. Furthermore, in previous methods in standard recombinant techniques, each clone expressing antibody needs to be screened individually. The present application provides for the selection of clones expressing antibodies with desired properties and thus only requires screening of clones from an enriched pool.

Because a rgdp (eg a pAb) is a novel structure that displays a member of a specific binding pair (eg. an antibody of monoclonal antigen-binding specificity) at the surface of a relatively simple replicable structure also containing the genetic information encoding the member, rgdps eg pAbs, that bind to the complementary member of the specific binding pair (eg antigen) can be recovered very efficiently by either eluting off the complementary member using for example diethylamine, high salt etc and infecting suitable bacteria, or by denaturing the structure, and specifically amplifying the sequences encoding the member using PCR. That is, there is no necessity to refer back to the original bacterial clone that gave rise to the pAb.

For some purposes, for example immunoprecipitation

and some diagnostic tests, it is advantageous to use polyclonal antibodies or antibody fragments. The present invention allows this to be achieved by either selection of an enriched pool of pAbs with desired properties or by mixing individually isolated clones with desired properties. The antibodies or antibody fragments may then be expressed in soluble form if desired. Such a selected polyclonal pAb population can be grown from stocks of phage, bacteria containing phagemids or bacteria expressing soluble fragments derived from the selected polyclonal population. Thus a reagent equivalent to a polyclonal antiserum is created which can be replicated and routinely manufactured in culture without use of animals.

SELECTION FORMATS AND AFFINITY MATURATION

Individual rgdps eg pAbs expressing the desired specificity eg for an antigen, can be isolated from the complex library using the conventional screening techniques (e.g. as described in Harlow, E., and Lane, D., 1988, supra Gherardi, E et al. 1990. J. Immunol. meth. 126 p61-68).

The applicants have also devised a series of novel selection techniques that are practicable only because of the unique properties of rgdps. The general outline of some screening procedures is illustrated in figure 2 using pAbs as an example type of rgdp.

The population/library of pAbs to be screened could be generated from immunised or other animals; or be created in vitro by mutagenising pre-existing phage antibodies (using techniques well-known in the art such as oligonucleotide directed mutagenesis (Sambrook, J., et al., 1989 Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press). This population can be screened in one or more of the formats described below with reference to figure 2, to derive those individual pAbs whose antigen binding properties are different from sample c.

Binding Elution

Figure 2(i) shows antigen (ag) bound to a solid surface (s) the solid surface (s) may be provided by a petri dish, chromatography beads, magnetic beads and the like. The population/library of pAbs is then passed over the ag, and those individuals p that bind are retained after washing, and optionally detected with detection system d. A detection system based upon anti-fd antisera is illustrated in more detail below in example 4. If samples of bound population p are removed under increasingly stringent conditions, the binding affinity represented in each sample will increase. Conditions of increased stringency can be obtained, for example, by increasing the time of soaking or changing the pH of the soak solution, etc.

Competition

Referring to figure 2(ii) antigen ag can be bound to a solid support s and bound to saturation by the original binding molecule c. If a population of mutant pAb (or a set of unrelated pAbs) is offered to the complex, only those that have higher affinity for antigen ag than c will bind. In most examples, only a minority of population c will be displaced by individuals from population p. If c is a traditional antibody molecule, all bound material can be recovered and bound p recovered by infecting suitable bacteria and/or by use of standard techniques such as PCR.

An advantageous application is where ag is used as a receptor and c the corresponding ligand. The recovered bound population p is then related structurally to the receptor binding site/and or ligand. This type of specificity is known to be very useful in the pharmaceutical industry.

Another advantageous application is where ag is an antibody and c its antigen. The recovered bound population p is then an anti-idiotype antibody which have numerous uses in research and the diagnostic and pharmaceutical industries.

At present it is difficult to select directly for anti-idiotype antibodies. pAbs would give the ability to do this directly by binding pAb libraries (eg a naive library) to B cells (which express antibodies on their surface) and isolating those phage that bound well.

In some instances it may prove advantageous to pre-select population p. For example, in the anti-idiotype example above, p can be absorbed against a related antibody that does not bind the antigen.

However, if c is a pAb, then either or both c and p can advantageously be marked in some way to both distinguish and select for bound p over bound c. This marking can be physical, for example, by pre-labelling p with biotin; or more advantageously, genetic. For example, c can be marked with an EcoB restriction site, whilst p can be marked with an EcoK restriction site (see Carter, P. et al., 1985, Nucl. Acids Res. 13, 4431-4443). When bound p+c are eluted from the antigen and used to infect suitable bacteria, there is restriction (and thus no growth) of population c (i.e. EcoB restricting bacteria in this example). Any phage that grew, would be greatly enriched for those individuals from p with higher binding affinities. Alternatively, the genetic marking can be achieved by marking p with new sequences, which can be used to specifically amplify p from the mixture using PCR.

Since the bound pAbs can be amplified using for example PCR or bacterial infection, it is also possible to rescue the desired specificity even when insufficient

individuals are bound to allow detection via conventional techniques.

The preferred method for selection of a phage displaying a protein molecule with a desired specificity or affinity will often be elution from an affinity matrix with a ligand (eg example 21). Elution with increasing concentrations of ligand should elute phage displaying binding molecules of increasing affinity. However, when eg a pAb binds to its antigen with high affinity or avidity (or another protein to its binding partner) it may not be possible to elute the pAb from an affinity matrix with molecule related to the antigen. Alternatively, there may be no suitable specific eluting molecule that can be prepared in sufficiently high concentration. In these cases it is necessary to use an elution method which is not specific to eg the antigen-antibody complex. Some of the non-specific elution methods generally used reduce phage viability for instance, phage viability is reduced with time at pH12 (Rossomando, E.F. and Zinder N.D. J. Mol.Biol. 36 387-399 1968). There may be interactions between eg antibodies and affinity matrices which cannot be disrupted without completely removing phage infectivity. In these cases a method is required to elute phage which does not rely on disruption of eg the antibody - antigen interaction. A method was therefore devised which allows elution of bound pAbs under mild conditions (reduction of a dithiol group with dithiothreitol) which do not disrupt phage structure (example 47).

This elution procedure is just one example of an elution procedure under mild conditions. A particularly advantageous method would be to introduce a nucleotide sequence encoding amino acids constituting a recognition site for cleavage by a highly specific protease between the foreign gene inserted, in this instance a gene for an antibody fragment, and the sequence of the remainder of gene III. Examples of such highly specific proteases are Factor X and thrombin. After binding of the phage to an affinity matrix and elution to remove non-specific binding phage and weak binding phage, the strongly bound phage would be removed by washing the column with protease under conditions suitable for digestion at the cleavage site. This would cleave the antibody fragment from the phage particle eluting the phage. These phage would be expected to be infective, since the only protease site should be the one specifically introduced. Strongly binding phage could then be recovered by infecting eg. E.coli TG1 cells.

An alternative procedure to the above is to take the affinity matrix which has retained the strongly bound pAb and extract the DNA, for example by boiling in SDS solution. Extracted DNA can then be used to directly

transform E.coli host cells or alternatively the antibody encoding sequences can be amplified, for example using PCR with suitable primers such as those disclosed herein, and then inserted into a vector for expression as a soluble antibody for further study or a pAb for further rounds of selection.

Another preferred method for selection according to affinity would be by binding to an affinity matrix containing low amounts of ligand.

If one wishes to select from a population of phages displaying a protein molecule with a high affinity for its ligand, a preferred strategy is to bind a population of phage to an affinity matrix which contains a low amount of ligand. There is competition between phage, displaying high affinity and low affinity proteins, for binding to the ligand on the matrix. Phage displaying high affinity protein is preferentially bound and low affinity protein is washed away. The high affinity protein is then recovered by elution with the ligand or by other procedures which elute the phage from the affinity matrix (example 35 demonstrates this procedure).

In summary then, for recovery of the packaged DNA from the affinity step, the package can be simply eluted, it can be eluted in the presence of a homologous sbp member which competes with said package for binding to a complementary sbp member; it could be removed by boiling, it could be removed by proteolytic cleavage of the protein; and other methods will be apparent to those skilled in the art eg. destroying the link between the substrate and complementary sbp member to release said packaged DNA and sbp member. At any rate, the objective is to obtain the DNA from the package so that it can be used directly or indirectly, to express the sbp member encoded thereby.

The efficiency of this selection procedure for pAbs and the ability to create very large libraries means that the immunisation techniques developed to increase the proportion of screened cells producing antibodies of interest will not be an absolute requirement. The technique allows the rapid isolation of binding specificities eg antigen-binding specificities, including those that would be difficult or even unobtainable by conventional techniques, for example, catalytic or anti-idiotypic antibodies. Removal of the animal altogether is now possible, once a complete library of the immune repertoire has been constructed.

The novel structure of the pAb molecule can be used in a number of other applications, some examples of which are:

Signal Amplification

Acting as a novel molecular entity in itself, rgdps eg pAbs combine the ability to bind a specific molecule eg antigen with amplification, if the major coat protein

is used to attach another moiety. This moiety can be attached via immunological, chemical, or any other means and can be used, for example, to label the complex with detection reagents or cytotoxic molecules for use in vivo or in vitro.

Physical Detection

The size of the rgdps eg pAbs can be used as a marker particularly with respect to physical methods of detection such as electron microscopy and/or some biosensors, e.g. surface plasmon resonance.

Diagnostic Assays

The rgdps eg pAbs also have advantageous uses in diagnostic assays, particularly where separation can be effected using their physical properties for example centrifugation, filtration etc.

In order that the invention is more fully understood, embodiments will now be described in more detail by way of example only and not by way of limitation with reference to the figures described below.

Figure 1 shows the basic structure of the simplest antibody molecule IgG.

Figure 2 shows schematically selection techniques which utilise the unique properties of pAbs; 2i) shows a binding/elution system; and (2ii) shows a competition system (p=pAb; ag=antigen to which binding by pAb is required; c=competitor population e.g. antibody, pAb, ligand; s=substrate (e.g. plastic beads etc); d=detection system.

Figure 3 shows the vector fd-tet and a scheme for the construction of vectors, fdTPs/Bs (for insertion of VH coding sequences) and fdTPs/Xh for the insertion of scFv coding sequences.

Figure 4 shows the nucleotide sequences for the oligonucleotides and vectors. All sequences are drawn 5' to 3' and are numbered according to Beck et al., 1978, Nucl. Acid Res., 5: 4495-4503. 4.1 shows the sequences of the oligonucleotides used for mutagenesis (oligo's 1 and 2) or sequencing (oligo 3). The sequences shown were synthesized on an Applied Biosystems, oligonucleotide synthesizer and are complementary to the single stranded form of fd-tet (they are in the anti-sense form with respect to gene III). 4.2 shows the sequences of the various constructs around the gene III insertion site. These sequences are drawn in the sense orientation with respect to gene III; (A) fd-tet (and fdTδBst) (B) fdTPs/Bs and (C) fdTPs/Xh. The key restriction enzyme sites are shown along with the immunoglobulin amino acids contributed by the vectors, (amino acid single letter code is used, see Harlow, E., and Lane, D., 1988 supra.).

Figure 5 shows the nucleotide and amino acid sequences for scFv in the vector scFvD1.3 myc. This gives the sequence of the anti-lysozyme single chain Fv

and surrounding sequences in scFvD1.3 myc, showing the N-terminal pel B signal peptide sequence and the C-terminal myc tag sequence (Ward, E.S., et al., 1989, supra.). Also shown is the peptide sequence linking the VH and VL regions. The amino acid sequence is represented above the nucleotide sequence by the single letter code, see Harlow, E., and Lane D., 1988 supra.

Figure 6 shows the binding of pAbs to lysozyme and the effect of varying the amount of supernatant. Each point is the average of duplicate samples. Lysozyme was coated at 1 mg/ml in 50 mM $NaHCO_3$.

Figure 7 shows the effect of varying the coating concentration of lysozyme or bovine serum albumin on the binding of pAbs to lysozyme in graphical form. Each point is the average of duplicate samples.

Figure 8 shows the sequence around the cloning site in gene III of fd-CAT2. Restriction enzyme sites are shown as well as the amino acids encoded by antibody derived sequences. These are flanked at the 5' end by the gene III signal peptide and at the 3' end by 3 alanine residues (encoded by the Not 1 restriction site) and the remainder of the mature gene III protein. The arrow shows the cleavage site for cutting of the signal peptide.

Figure 9 shows the binding of pAb (1.3) to lysozymes. Binding of phage as detected by ELISA to (a) hen egg-white lysozyme (HEL) (b) turkey egg-white lysozyme (TEL), (c) human lysozyme (HUL), (d) bovine serum albumin (BSA). A further control of (e) fdTPs/Bs to HEL.

Figure 10 shows a map of FabD1.3 in pUC19.

Figure 11 shows the ELISA results providing a comparison of lysozyme-binding by phage-Fab and phage-scFv. Vector=fdCAT2 (example 5); fdscFv(OX)=pAbNQ11 (Example 9); fdVHCH1 (D1.3)=grown in normal cells (i.e. no L chain, see example 7); fdFab(D1.3) i.e. fdVHCH1 (D1.3) grown in cells containing D1.3 L chain; fdscFv (D1.3)=pAbD1.3.

Figure 12 shows oligonucleotide probing of affinity purified phage. 10^{12} phage in the ratio of 1 pAb (D1.3) in 4 x 10^4 fdTPS/Bs phages were affinity purified and probed with an oligonucleotide specific for pAb (D1.3) A is a filter after one round of affinity purification (900 colonies total) and B is a filter after two rounds (372 colonies total).

Figure 13 shows the sequence of the anti-oxazolone antibody fragment NQ11 scFv. The sequence contributed by the linker is shown in the lower case. The sequence for VH is before the linker sequence and the sequence for VL is after the linker.

Figure 14 shows the ELISA results for binding pAb NQ11 and pAb D1.3 and vector fdTPs/xh to specified

antigens.

Figure 15 shows the sequence surrounding the phoA insertion in fd-phoAla166. The restriction sites used for cloning are shown, as well as the amino acids encoded by phoA around the insertion site. The first five amino acids of the mature fusion come from gene III.

Figure 16(1) shows the structure of gene III and the native BamHI site into which a scFv coding sequence was inserted in example 13 and figure 16(2) shows the natural peptide linker sites A and B for possible insertion of scFv coding sequences.

Figure 17 shows schematically the protocol for PCR assembly of mouse VH and VLK repertoires for phage display described in example 14.

Figure 18 shows examples of the final products obtained with the procedure of example 14. Lanes a and b show the products of the initial PCR using heavy and light chain primers respectively; lane c shows the complete assembled 700bp product before final digestion with NotI and ApaL1; M1, M2 markers Φ174 Hae III digest and 123 base pair ladder (BRL Limited, P.O. Box 35, Washington Road, Paisley, Scotland) respectively.

Figure 19 shows the binding of ^{125}I-PDGF-BB to fd h-PDGFB-R phage in immunoprecipitation assay and comparison to fdTPs/Bs and no phage controls; binding is expressed as a percentage of the total ^{125} I-PDGF-BB added to the incubation.

Figure 20 shows the displacement of ^{125}I-PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Binding is expressed as a percentage of the total ^{125}I-PDGF-BB added to the incubation.

Figure 21 shows the displacement of ^{125}I-PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Non-specific binding of ^{125}I-PDGF-BB to vector phage fdTPs/Bs in the absence of added unlabelled PDGF was deducted from each point.

Figure 22 shows the results of an ELISA of lysozyme binding by pCAT-3 scFv D1.3 phagemid in comparison with pCAT-3 vector (both rescued by M13K07) and fdCAT2 scFv D1.3 as described in example 17. The ELISA was performed as described in example 6 with modifications detailed in example 18.

Figure 23 shows the digestion pattern seen when individual clones, selected at random from a library of single chain Fv antibody genes derived from an immunised mouse; are digested with BstN1.

Figure 24 shows VH and VK gene sequences derived from the combinatorial library in example 21 and the hierarchical library in example 22.

Figure 25 shows a matrix of ELISA signals for clones

derived from random combinatorial library. Designation of the clones is as in figure 24. The number of clones found with each combination is shown by the numerals.

Figure 26 shows a) the phagemid pHEN1 a derivative of pUC119 described in example 24; and b) the cloning sites in the phagemid pHEN.

Figure 27. The antibody constructs cloned into fd-CAT2 and pHEN1 for display on the surface of phage. Constructs I, II, III and IV were cloned into both fd-CAT2 (as ApaLI-NotI fragments) and pHEN1 (as SfiI-NotI fragments) and pHEN1 (as SfiI-NotI fragments). All the constructs contained the heavy chain (VH) and light chain (VK) variable regions of the mouse anti-phOx antibody NQ10.12.5. The constant domains were human CK and CH1 (γ 1 isotype).

Figure 28. Three ways of displaying antibody fragments on the surface of phage by fusion to gene III protein.

Figure 29. Western blot of supernatant taken from pHEN1-II(+) or pHEN1(-) cultures in E.coli HB2151, showing secretion of Fab fragment from pHEN1-II only. The anti-human Fab detects both H and L chain. Due to the attached c-myc tag, the L chain, highlighted by both anti-c-myc tag and anti-human CK antisera, is slightly larger (calculated Mr 24625) than the H chain (calculated Mr23145).

Figure 30 is a plot showing the effect of lysozyme dilution on ratio of ELISA signals obtained using pAbD1.3 or soluble scFv D1.3.

Figure 31 is a plot showing the effect of lysozyme dilution on ELISA signals obtained using fdTscFvD1.3 and soluble scFvD1.3.

Figure 32 is a plot showing positive results from an ELISA screen of phage displaying scFv fragments derived from the cell line 013 which express a monoclonal antibody directed against oestriol.

Figure 33 is a plot showing positive results from an ELISA screen of phage displaying scFv fragments derived from the cell line 014 which express a monoclonal antibody directed against oestriol.

Figure 34 is a Western Blot showing expression of the alkaline phosphatase-gene 3 fusion. 16µl of 50 fold concentrate of each phage sample was detected on western blots with either anti-gene 3 antiserum (e-f) or with anti-alkaline phosphatase antiserum (c-f)

a) fd-phoAla166 grown in TG1 cells
b) fd-phoAla166 grown in KS272 cells
c) fdCCAT2 grown in TG1 cells
d) fdCAT2 grown in TG1 cells, mixed with 13 ng of purified alkaline phosphatase
e) fd-phoAla166 grown in TG1 cells
f) fdCAT2 grown in TG1 cells.

Figure 35 is a Western Blot showing ultrafiltration of phage-enzyme 100µl of 50 fold concentrate of phage (representing 5mls of culture supernatant) was centrifuged through ultrafiltration membranes with nominal molecular weight retention of 300,000 daltons. Western blots of flow through and retentate fractions were detected with anti-alkaline phosphatase antiserum. The equivalent of 800µl of original culture supernatant was run on the gel.

A. Phage were grown in TG1 cells. a) fd-phoAla166 before ultrafiltration (short exposure). b) fd-phoAla166 before ultrafiltration. c) fd-phoAla166 material retained on ultrafiltration membrane.

B. Phage were grown in KS272 cells. a) fd-phoAla166 before ultrafiltration. b) fd-phoAla166 material retained on ultrafiltration membrane. c) fdCAT2. d) fdCAT2 mixed with purified alkaline phosphatase before ultrafiltration. e) Retentate from sample d. f) Flow through from sample d.

Figure 36 Electrophoresis of samples from stages of a Fab assembly. Samples from different stages in the PCR Fab assembly process described in example 33 were subjected to electrophoresis on a 1% TAE-agarose gel. Samples from a comparable scFv assembly process (as in example 14) are shown for comparison. Samples left to right are:

M	=	Markers
VHCH1	=	sequences encoding VHCH1 domains amplified by PCR
VKCK	=	sequences encoding VKCK domains amplified by PCR
-L	=	Fab assembly reaction performed in absence of linker
+L	=	Fab PCR assembly reaction product VHCH1 plus VKCK plus linker
M	=	Markers
VK	=	sequences encoding VK domain amplified by PCR
VL	=	sequences encoding VH domains amplified by PCR
-L	=	scFv assembly reaction in absence of linker
+L	=	scFv assembly reaction in presence of linker
M	=	Markers

Figure 37. Comparison of ELISA signals with scFv D1.3 cloned in fd-CAT2 (fd) or pCAT-3. pCAT-3 scFv1.3 has been rescued with M13KO7 (KO7). M13KO7ΔgIII No 3 (gIII No 3) or M13KO7 gIIIΔNo 2 (g111No2). Phage antibodies are compared at 10 times (10x) 1 times (1x) or 0.1 times

(0.1x) concentrations relative to concentration in the supernatant after overnight growth. The fdCAT2 and pCAT-3 non-recombinant vector signals were <0.01 at 10x concentration. M13KO7 gIIIΔNo 1 did not rescue at all, as judged by no signal above background in this ELISA.

Figure 38. Western blot of PEG precipitated phage used in ELISA probed with anti-g3p. Free g3p and the g3p-scFvD1.3 fusion bands are arrowed.
Sample 1 - fd scFvD1.3
Sample 2 - pCAT3 vector
Sample 3 - pCAT3 scFvD1.3 rescued with M13KO7, no IPTG
Sample 4 - pCAT3 scFvD1.3 rescued with M13KO7, 50μM IPTG
Sample 5 - pCAT3 scFvD1.3 rescued with M13KO7, 100μM IPTG
Sample 6 - pCAT3 scFvD1.3 rescued with M13KO7 gIIIΔNo3 (no IPTG)
Sample 7 - pCAT3 scFvD1.3 rescued with M13KO7 gIIIΔ No 2 (no IPTG)

Panel A samples contain the equivalent of 8μl of phagemid culture supernatant per track, and 80μl of the fd supernatant (10-fold lower phage yield than the phagemid). Panel B phagemid samples are those used in panel A at a five-fold higher sample loading (equivalent to 40μl of culture supernatant per track) to enable visualisation of the fusion band in samples rescued with parental M13KO7.

Figure 39 is a graph showing fdCAT2scFvD1.3 enrichment produced from a mixture of fdCAT2scFvD1.3 and fdCAT2TPB4 by one round of panning.

Figure 40 is a graph showing fdCAT2scFvD1.3 enrichment produced from a mixture of fdCAT2scFvD1.3 and fdCAT2TPB1 by one round of panning.

Figure 41. Western blot of phage proteins of fdCAT2(1) and fd-tet-SNase(2) with anti-g3p antiserum. Marker molecular weights bands are indicated(kD).

Figure 42. Nuclease assay of soluble SNase (3 ng)(A-1),fd-tet-SNase(4 x 10^9TU,(B-1),fd-CAT2(2 x 10^{10}TU)(C-1) and of a PEG-precipitated fdCAT2 and SNase mixture(2 x 10^{10}TU and 0.7ug)(D-1) in a 10-fold dilution series (1 to 3 or 4). Marker (M) is a HindIII digest of λ-DNA(New England Biolabs).

Figure 43. ELISA signals obtained with fd-tet, fd-CD4-V1 and fd-CD4-V1V2. In each group of three, the samples are left to right phage concentrate(SN); phage concentrate plus soluble CD4(SN + sCD4); phage concentrate plus gp 120 (SN + gp 120).

Figure 44. shows the DNA sequence of scFv B18 (anti-NP).

Figure 45 shows a map of the insert of sequences encoding FvD1.3 present in fd-tet FvD1.3 (example 39). rbs designates the ribosome binding site. Gene III is now shown in its full length.

Figure 46. shows an ELISA assay of phages displaying

FvD1.3 or scFvD1.3 by binding to plates coated with lysogyme. Signals obtained at various dilution factors are shown. FvD1.3 (ΔS-Stuffer) which does not express Fv was used as a control.

Figure 47. shows a schematic representation of steps involved in the PCR assembly of nucleotide sequences encoding human Fab fragments. Details are in example 40.

Figure 48. shows A. a map of plasmid pJM1-FabD1.3 which is used for the expression of soluble human Fab fragments and as a template for the synthesis of linker DNA for Fab assembly. B. a schematic representation of sequences encoding a Fab construct. C. The sequence of DNA template for the synthesis of linker DNA for Fab assembly.

Figure 49. shows a schmatic representation of steps involved in the PCR assembly of nucleotide sequences encoding human scFv fragments. Details are in example 42.

Figure 50. ELISA assay of phage antibodies using plates coated with turkey egg lysogyme. Two clones B1 and A4 are shown derived by mutagenesis and selection from pAbD1.3 (example 45). Concentration (x axis) refers to the concentration of phage for each sample relative to the concentration in culture supernatant. B1 has raised binding to turkey egg lysogyme compared to D1.3. A4 has reduced binding to hen egg lysogyme compared to D1.3.

Figure 51. ELISA of phage antibodies binding to HEL and TEL. Clone 1 is fdCAT2scFvD1.3. Clones 2 to 10 were obtained from the library (example 46) after selection. The background values as defined by binding of these clones to BSA were subtracted.

Figure 52. shows the DNA sequence of the light chains D1.3 M1F and M21 derived by selection from a hierarchical library in example 46.

Figure 53 shows a Fv lambda expression vector (example 48) derived from pUC119. It contains the rearranged lambda1 germ line gene. The heavy and light chain cassettes each contain a ribosome binding site upstream of the pel B leader (Restriction sites shown as: H=Hind III; Sp=SphI; B=BamHI, E=EcoRI.

Materials and Methods

The following procedures used by the present applicants are described in Sambrook, J. et al., 1989 supra.: restriction digestion, ligation, preparation of competent cells (Hanahan method), transformation, analysis of restriction enzyme digestion products on agarose gels, purification of DNA using phenol/chloroform, 5'-end labelling of oligonucleotides, filter screening of bacterial colonies, preparation of 2xTY medium and plates, preparation of tetracycline stock solutions, PAGE of proteins, preparation of phosphate buffered saline.

All enzymes were supplied by New England Biolabs (CP Laboratories, PO Box 22, Bishop's Stortford, Herts., England) and were used according to manufacturer's instructions unless otherwise stated.

The vector fd-tet (Zacher, A.N. et al., 1980, supra) was obtained from the American Type Culture Collection (ATCC No. 37000) and transformed into competent TG1 cells (genotype: K12δ (lac-pro), sup E, thi, hsdD5/F traD36, pro A+B+, Lac I^q, lac δM15).

Viral particles were prepared by growing TG1 cells containing the desired construct in 10 to 100 mls 2xTY medium with 15 µg/ml tetracycline for 16-24 hours. The culture supernatant was collected by centrifugation for 10 mins at 10,000 rpm in an 8 x 50 ml rotor, Sorval RC-5B centrifuge. Phage particles were precipitated by adding 1/5th volume 20% polyethylene glycol (PEG)/2.5M NaCl and leaving at 4°C for 1 hour. These were spun for 15 minutes as described above and the pellets resuspended in 10 mM Tris/HCl pH 8, 1mM EDTA to 1/100th of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 minutes in a microcentrifuge. Single stranded DNA for mutagenesis or sequencing was prepared from concentrated phage according to Sambrook, J., et al., 1989,, supra.

Index of Examples

Example 1 Design of Insertion Point Linkers and Construction of Vectors

This example covers the construction of two derivatives of the phage vector fd-tet: a) fdTPs/Bs for the insertion of VH coding sequences; and b) fdTPs/Xh for the insertion of scFv coding sequences. The derivative vectors have a new BstEII site for insertion of sequences.

Example 2 Insertion of Immunoglobulin Fv Domain into Phage

This example covers the insertion of scFv coding sequences derived from an anti-lysozyme antibody D1.3 into fdTPs/Xh to give the construct fdTscFvD1.3.

Example 3 Insertion of Immunoglobulin VH Domain into Phage

This example covers the insertion of VH coding sequences derived from an anti-lysozyme antibody D1.3 into fdTPs/Bs to give the construct fdTVHD1.3.

Example 4 Analysis of Binding Specificity of Phage Antibodies

This example investigates the binding specificities of the constructs fdTscFvD1.3 and fdTVHD1.3.

Example 5 Construction of fdCAT2

This example covers the construction of the derivative fdCAT2 of the phage vector fdTPs/Xh. The derivative has restriction sites for enzymes that cut DNA infrequently.

Example 6 Specific Binding of Phage Antibody (pAb) to Antigen

This example shows the binding of pAb fdTscFvD1.3 to lysozyme by ELISA.

Example 7 Expression of FabD1.3

This example concerns the display of an antibody Fab fragment at the phage surface. The VH-CH1 chain is expressed by fdCAT2. The VL-CL chain is expressed by pUC19 in a bacterial host cell also infected with fdCAT2.

Example 8 Isolation of Specific, Desired Phage from a Mixture of Vector Phage

This example shows how a phage (e.g. fdTscFvD1.3) displaying a binding molecule can be isolated from vector phage by affinity techniques.

Example 9 Construction of pAb Expressing Anti-Hapten Activity

This example concerns the insertion of scFv coding sequences derived from the anti-oxazolone antibody NQ11 into fdTPs/Xh to generate the construct pAbNQ11. The example shows the binding of pAbNQ11 to oxazalone by ELISA.

Example 10 Enrichment of pAbD1.3 from Mixtures of other pAbs by Affinity Purification

This example shows how a phage (eg. pAbD1.3) displaying one sort of biding molecule can be isolated from phage (e.g. pAbNQ11) displaying another sort of binding molecule by affinity techniques.

Example 11 Insertion of a Gene Encoding an Enzyme (Alkaline Phosphate) into fdCAT2

This example concerns the invention of coding sequences for an enzyme into the vector fdCAT2 to give the phage enzyme, fdphoAla116.

Example 12 Measuring Enzyme Activity Phage - Enzyme

This example shows the functionality of an enzyme (alkaline phosphatase) when displayed at the phage surface (fdphoAla166).

Example 13 Insertion of Binding Molecules into Alternative Sites in the Phage

This example covers the insertion of scFv coding sequences derived from a) the anti-lysozyme antibody D1.3; and b) the anti-oxazalone antibody NQ11 into a BamH1 site of fdTPs/Xh to give the constructs fdTBam1 having an NQ11 insert.

Example 14 PCR Assembly of Mouse VH and VLK Repertoires for Phage Display

This example concerns a system for the display on phage of all VH and VLK repertoires encoded by a mouse. The system involves the following steps. 1) Preparation of RNA from spleen. 2) Preparation of cDNA from the RNA 3) Use of primers specific for antibody sequences to PCR amplify all VH and VLK cDNA coding sequences 4) Use of PCR to create a linker molecule from linking pairs of VH

and VLK sequences 5) Use of PCR to assemble continuous DNA molecules each comprising a VH sequence, a linker and a VLK sequence. The specific VH/VLK combination is randomly derived 6) Use of PCR to introduce restriction sites.

Example 15. Insertion of the Extracellular Domain of a Human Receptor for Platelet Derived Growth Factor (PDGF) Isoform BB into fdCAT2

This example concerns the insertion of coding sequences for the extracellular domain of the human receptor for PDGF into the vector fdCAT2 to give the construct fdhPDGFBR.

Example 16. Binding of 125 I-PDGF-BB to the Extracellular Domain of the Human Receptor for PDGF Isoform BB Displayed on the Surface of fd Phage. Measured using an Immunoprecipitation Assay.

This example shows that the human receptor PDGF Isoform BB is displayed on the surface of the phage in a form which has the ability to bind its ligand.

Example 17. Construction of Phagemid Containing Gene III Fused with the Coding Sequence for a Binding Molecule.

This example concerns the construction of two phagemids based on pUC119 which separately contain gene III from fdCAT2 and the gene III scFv fusion fdCAT2seFvDI.3 to generate pCAT2 and pCAT3 scFvDI.3 respectively.

Example 18. Rescue of Anti-Lysozyme Antibody Specificity from pCAT3scFvD1.3 by M13KO7

This example describes the rescue of the coding sequence for the gene IIIscFv fusion from pCAT3scFvD1.3 by M13MO7 helper phage growth, phage were shown to be displaying scFv anti-lypozyme activity by ELISA.

Example 19. Transformation Efficiency of PCAT-3 and pCAT-3 scFvD1.3 Phagemids

This example compared the efficiency of the phagemids pVC119, pCAT-3 and pCAT3scFvD1.3 and the phage fdCAT2 to transform E.coli.

Example 20 PCR Assembly of a Single Chain Fv Library from an Immunised Mouse

This example concerns a system for the display on phage of scFv (comprising VH and VL) from an immunised mouse using the basic technique outlined in example 14 (cDNA preparation and PCR assembly of the mouse VH and VLK repertoires) and ligating the PCR assembled sequences into fdCAT2 to create a phage library of 10^5 clones. Testing of 500 clones showed that none showed specificity against phox.

Example 21. Selection of Antibodies Specific for 2-phenyl-5-oxazolone from a Repertoire from an Immunised Mouse.

This example shows that phage grown from the library established in example 20 can be subjected to affinity selection using phOX to select those phage displaying

scFv with the desired specificity.

Example 22. Generation of Further Antibody Specificities by the Assembly of Hierarchial Libraries.

This example concerns the construction of hierarchial libraries in which a given VH sequence is combined with the complete VLK repertoire and a given VLK sequence is combined with the complete VH repertoire and selection from these libraries of novel VH and VL pairings.

Example 23. Selection of Antibodies Displayed on Bacteriophage with Different Affinities for 2-phenyl-5-oxazolone using Affinity Chromatography

This example concerns the separation by affinity techniques of phages displaying scFv fragments with differing binding affinities for a given antigen.

Example 24. Construction of Phagemid pHEN1 for the Expression of Antibody Fragments Expressed on the surface of Bacteriophage following Superinfection

This example concerns the construction of the phagemid pHEN1 derived from pUC119. pHEN1 has the features shown in Fig. 26.

Example 25. Display of Single Chain Fv and Fab Fragments Derived from the Anti-Oxazolone Antibody NQ 10.12.5 on Bacteriophage fd using pHEN1 and fdCAT2.

This example describes the display of scFv and Fab fragment with a specificity against phOx on the surface of a bacteriophage. For display of scFv the phagemid pHEN1 comprises the sequences encoding scFv (VH and VL) for rescue by either the phages VSM13 or fdCAT2. For display of Fab the phage fdCAT2 comprises the sequence for either the H or L chain as a fusion with g3p and the phagemid pHEN1 comprises the sequence for the appropriate H or L chain partner.

Example 26. Rescue of Phagemid Encoding a Gene III protein Fusion with Antibody Heavy or Light Chains by Phage Encoding the Complementary Antibody Displayed on Phage and the Use of this Technique to make Dual Combinatorial Libraries

This example covers the use of phage antibodies encoding the antibody heavy or light chain to rescue a phagemid encoding a gene 3 protein fusion with the complementary chain and the assay of Fab fragments displayed on phage in ELISA. The use of this technique in the preparation of a dual combinatorial library is discussed.

Example 27 Induction of Soluble scFv and Fab Fragments using Phagemid pHEN1

This example covers the generation of soluble scFv and Fab fragments from gene III fusions with sequences encoding these fragments by expression of clones in pHEN1 in an E.coli strain which does not suppress amber mutations.

Example 28 Increased Sensitivity in ELISA of Lysozyme

using fdTscFvD1.3 as Primary Antibody compared to Soluble scFvD1.3

This example covers the use of fdTscFvD1.3 in ELISA showing that lower amounts of lysozyme can be detected with phage antibody fdTscFvD1.3 than with soluble scFvD1.3.

Example 29 Direct Rescue and Expression of Mouse Monoclonal Antibodies as Single Chain Fv Fragments on the Surface of Bacteriophage fd

This example covers the display on phage as functional scFv fragments of two clones directly derived from cells expressing monoclonal antibodies directed against oestriol. Both clones were established to be functional using ELISA.

Example 30 Kinetic Properties of Alkaline Phosphatase Displayed on the Surface of Bacteriophage fd

This example concerns the demonstration that the kinetic properties of an enzyme, alkaline phosphatase, displayed on phage are qualitatively similar to those of the same enzyme when in solution.

Example 31 Demonstration using Ultrafiltration that Cloned Alkaline Phosphatase Behaves as Part of the Virus Particle

This example concerns the construction of the phage enzyme fdphoArg166 and the demonstration that both the fusion protein made and the catalytic activity observed derive from the phage particle.

Example 32 Affinity Chromatography of Phage Alkaline Phosphatase

This example concerns the binding of alkaline phosphatase displayed on phage to an arsenate-Sepharose affinity column and specific elution of these phage using the reaction product, phosphate.

Example 33 PCR Assembly of DNA Encoding the Fab Fragment of an Antibody Directed against Oxazolone

This example covers the construction of a DNA insert encoding a Fab fragment by separate amplification of heavy and light chain DNA sequences followed by assembly. The construct was then inserted into the phage vector fdCAT2 and the phagemid vector pHEN1 and the Fab fragment displayed on the surface was shown to be functional.

Example 34 Construction of a Gene III Deficient Helper Phage

This example describes the construction of a helper phage derived from M13KO7 by deleting sequences in gene III. Rescue of pCAT3-scFvD1.3 is described. The scFvD1.3 is expressed at a high level as a fusion using the deletion phage, equivalent to expression using fdCAT2-scFvD1.3.

Example 35 Selection of bacteriophage expressing scFv fragments directed against lysozyme from mixtures according to affinity using a panning procedure

This example concerns the selection of bacteriophage according to the affinity of the scFv fragment directed against lysozyme which is expressed on their surface. The phage of different affinities were bound to Petri dishes coated with lysozyme and, following washing, bound phage eluted using triethylamine. Conditions were found where substantial enrichment could be obtained for a phage with a 5-fold higher affinity than the phage with which it was mixed.

Example 36 Expression of Catalytically Active Staphylococcal Nuclease on the Surface of Bacteriophage fd

This example concerns the construction of a phage enzyme which expresses Staphylococcal nuclease and the demonstration that the phage enzyme retains nuclease activity.

Example 37 Display of the Two Aminoterminal Domains of Human CD4 on the Surface of fd Phage

This example covers the cloning of genes for domains of CD4, a cell surface receptor and member of the immunoglobulin superfamily, into bacteriophage fd. The receptor is shown to be functional on the surface of phage by binding to the HIV protein gp120.

Example 38 Generation and Selection of Mutants of an Anti-4-hydroxy-3-nitrophenylacetic acid (NP) Antibody expressed on Phage using Mutator strains

This example covers the introduction of mutations into a gene for an antibody cloned in phage by growth of the phage in strains which randomly mutate DNA due to defects in DNA replication. Several mutations are introduced into phage which can then be selected from parent phage.

Example 39 Expression of a Fv Fragment on the Surface of Bacteriophage by Non-Covalent Association of VH and VL domains

This example shows that functional Fv fragments can be expressed on the surface of bacteriophage by non-covalent association of VH and VL domains. The VH domain is expressed as a gene III fusion and the VL domain as a soluble polypeptide. Sequences allowing expression of these domains from the anti-lysozyme antibody D1.3 in this form were introduced into phage and the resulting displayed Fv fragment shown to be functional by ELISA.

Example 40 A PCR Based Technique for one step Cloning of Human V-genes as Fab Constructs

This example gives methods for the assembly of Fab fragments from genes for antibodies. Examples are given for genes for antibodies directed against Rhesus-D in a human hybridoma and a polyclonal lymphoblastic cell line.

Example 41 Selection of Phage Displaying a Human Fab Fragment directed against the Rhesus-D Antigen by binding to Cells displaying the Rhesus D Antigen on their Surface

This example concerns the construction of, and display of phage antibodies from, a phagemid encoding a human Fab fragment directed against the Rhesus D antigen. Phage displaying this antigen were then affinity selected from a background of phage displaying scFvD1.3 anti-lysozyme on the basis of binding to Rhesus-D positive red blood cells.

Example 42 A PCR Based Technique for One Step Cloning of Human scFv Constructs

This example describes the generation of libraries of scFv fragments derived from an unimmunized human. Examples are given of the preparation for phage display of libraries in phagemids of scFv fragments derived from IgG and IgM sequences.

Example 43 Isolation of Binding Activities from a Library of scFvs from an Unimmunized Human

This example describes the isolation, from the library of scFv fragments derived from IgM genes of an unimmunized human, of clones for phage antibodies directed against BSA, lysozyme and oxazolone. Selection was by panning or affinity chromatography and analysis of binding specificity by ELISA. Sequencing of the clones showed them to be of human origin.

Example 44 Rescue of human IgM library using helper phage lacking gene 3 (g3)

This example covers the isolation, from the library of scFv fragments of unimmunized human IgM genes, of clones of phage antibodies of clones for phage antibodies specific for thyroglobulin and oxazolone. In this example rescue was with M13K07gIII No3 (NCTC12478), a helper phage defective in gene III. Fewer rounds of selection appeared necessary for a phagemid library rescued with this phage compared to one rescued with M13K07.

Example 45 Alteration of Fine Specificity of scFvD1.3 displayed on Phage by Mutagenesis and Selection on Immobilized Turkey Lysozyme

This example covers the in vitro mutagenesis of pCATscFvD1.3 by replacement, with random amino acids, of residues known to be of importance in the preferential recognition of hen egg lysozyme over turkey egg lysozyme by scFvD1.3. Following selection for phage antibodies recognising turkey egg lysozyme by affinity chromatography, clones were analysed for specificity by ELISA. Two groups of clones were found with more equal recognition of hen and turkey lysozymes, one with increased ELISA signal with the turkey enzyme and one with reduced signal for the hen enzyme.

Example 46 Modification of the Specificity of an Antibody by Replacement of the VLK Domain by a VLK Library derived from an Unimmunised Mouse

This example shows that replacement of the VL domain

of scFvD1.3 specific for hen eggwhite lysozyme (HEL) with a library of VL domains allows selection of scFv fragments which bind also to turkey eggwhite lysozyme (TEL). The scFv fragments were displayed on phage and selection by panning on tubes coated with TEL. Analysis by ELISA showed clones with enhanced binding to TEL compared to HEL. Those with highest binding to TEL were sequenced.

Example 47 Selection of a Phage Antibody Specificity by binding to an Antigen attached to Magnetic Beads. Use of a Cleavable Reagent to allow Elution of Bound Phage under Mild Conditions

This examples covers the use of a cleavable bond in the affinity selection method to alow release of bound phage under mild conditions. pAbNQ11 was enriched approximately 600 fold from a mixture with pAbD1.3 by selection using biotinylated Ox-BSA bound to magnetic beads. The cleavage of a bond between BSA and the biotin allows elution of the phage.

Example 48 Use of Cell Selection to provide an Enriched Pool of Antigen Specific Antibody Genes, Application to reducing the Complexity of Repertoires of Antibody Fragments Displayed on the Surface of Bacteriophage

This example covers the use of cell selection to produce an enriched pool of genes encoding antibodies directed against 4-hydroxy-3-nitrophenylacetic acid and describes how this technique could be used to reduce the complexity of antibody repertoires displayed on the surface of bacteriophage.

Example 1

Design of Insertion Point Linkers and Construction of Vectors

The vector fd-tet has two BstEII restriction sites flanking the tetracycline resistance gene (fig 3). Since the strategy for inserting the VH fragments was to ligate them into a newly inserted BstEII site within gene III, it was advantageous to delete the original BstEII sites from fd-tet. This was achieved by digesting fd-tet with the restriction enzyme BstEII, filling-in the 5' overhangs and re-ligating to generate the vector fdTδBst. Digestion of fd-tet with BstEII (0.5 units/µl) was carried out in 1x KGB buffer (100 mM potassium glutamate, 23 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 µg/ml bovine serum albumin, 0.5 mM dithiothreitol (Sambrook, J., et al., 1989, supra.) with DNA at a concentration of 25 ng/µl. The 5' overhang was filled in, using 2x KGB buffer, 250 µM each dNTP's (Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks., UK.) and Klenow Fragment (Amersham International, Lincoln Place, Green End, Aylesbury, Bucks., UK) at 0.04 units/µl. After incubating for 1 hour at room temperature, DNA was extracted with

phenol/chloroform and precipitated with ethanol.

Ligations were carried out at a DNA concentration of 50ng/µl). Ligations were transformed into competent TG1 cells and plated onto TY plates supplemented with 15 µg/ml tetracycline. This selects for vectors where the gene for tetracycline resistance protein has reinserted into the vector during the ligation step. Colonies were picked into 25 mls of 2xTY medium supplemented with 15 µg/ml tetracycline and grown overnight at 37°C.

Double stranded DNA was purified form the resulting clones using the gene-clean II kit (Bio101 Inc., PO Box 2284, La Jolla, California, 92038-2284, USA.) and according to the small scale rapid plasmid DNA isolation procedure described therein. The orientation of 5 of the resulting clones was checked using the restriction enzyme ClaI. A clone was chosen which gave the same pattern of restriction by ClaI as fd-tet, but which had no BstE II sites.

In vitro mutagenesis of fdTδBst was used to generate vectors having appropriate restriction sites that facilitate cloning of antibody fragments downstream of the gene III signal peptide and in frame with the gene III coding sequence. The oligonucleotide directed mutagenesis system version 2 (Amersham International) was used with oligo 1 (figure 4) to create fdTPs/Bs (to facilitate cloning of VH fragments). The sequence offdTPs/Bs (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp., PO Box 22400, Cleveland, Ohio, 44122, UsA.) with oligo 3 (figure 4) as a primer.

A second vector fdTPs/Xh (to facilitate cloning of single chain Fv fragments) was generated by mutagenising fdTPs/Bs with oligo 2 according to the method of Venkitaraman, A.R., Nucl. Acid Res. 17, p 3314. The sequence of fdTPs/Xh (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp.) with oligo 3 as a primer.

Clearly, alternative constructions will be apparent to those skilled in the art. For example, M13 and/or its host bacteria could be modified such that its gene III could be disrupted without the onset of excessive cell death; the modified fd gene III, or other modified protein, could be incorporated into a plasmid containing a single stranded phage replication origin, such as pUC119, superinfection with modified phage such as KO7 would then result in the encapsulation of the phage antibody genome in a coat partially derived from the helper phage and partly from the phage antibody gene III construct.

The detailed construction of a vector such as fdTPs/Bs is only one way of achieving the end of a phage antibody. For example, techniques such as sticky feet cloning/mutagenesis (Clackson, T. and Winter, G. 1989

Nucl. Acids. Res., 17, p 10163-10170) could be used to avoid use of restriction enzyme digests and/or ligation steps.

Example 2.

Insertion of Immunoglobulin Fv Domain into Phage

The plasmid scFv D1.3 myc (gift from g. Winter and A. Griffiths) contains VH and VL sequences from the antibody D1.3 fused via a peptide linker sequence to form a single chain Fv version of antibody D1.3. The sequence of the scFv and surrounding sequences in scFvD1.3 myc is shown in figure 5.

The D1.3 antibody is directed against hen egg lysozyme (Harper, M. et al., 1987, Molec. Immunol. 24, 97-108) and the scFv form expressed in E.coli has the same specificity (A. Griffiths and G. Winter personal Communication).

Digestion of scFv D1.3 myc with PstI and XhoI (these restriction sites are shown on Fig. 5), excises a fragment of 693 bp which encodes the bulk of the scFv. Ligation of this fragment into fdTPs/Xh cleaved with PstI and XhoI gave rise to the construct fdTscFvD1.3 encoding the gene III signal peptide and first amino acid fused to the complete D1.3 scFv, followed by the mature gene III protein from amino acid 2.

The vector fdTPs/Xh was prepared for ligation by digesting with the PstI and XhoI for 2 hours followed by digestion with calf intestinal alkaline phosphatase (Boehringer Mannheim UK Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG) at one unit/ul for 30 minutes at 37°C. Fresh calf intestinal alkaline phosphatase was added to a final total concentration of 2 units/ul and incubated for a further 30 minutes at 37°C. The reaction was extracted three times with phenol/chloroform, precipitated with ethanol and dissolved in water. The insert from scFvD1.3 myc was excised with the appropriate restriction enzymes (PstI and XhoI) extracted twice with phenol/chloroform, precipitated with ethanol and dissolved in water. Ligations were carried out as described in example 1, except both vector and insert samples were at a final concentration of 5 ng/ul each. The formation of the correct construct was confirmed by sequencing as described in example 1.

To demonstrate that proteins of the expected size were produced, virions were concentrated by PEG precipitation as described above. The samples were prepared for electrophoresis as described in Sambrook J. et al 1989 supra. The equivalent of 2mls of supernatant was loaded onto an 18% SDS polyacrylamide gel. After electrophoresis, the gel was soaked in gel running buffer (50 mM tris, 380 mM Glycine, 0.1%SDS) with 20% methanol for 15 minutes. Transfer to nitrocellulose filter was executed in fresh 1x running buffer/20% methanol using

TE70 Semi Phor a semi-dry blotting apparatus (Hoeffer, 654 Minnesota Street, Box 77387, San Francisco, California 94107, USA.).

AFter transfer, the filter was blocked by incubation for 1 hour in a 2% solution of milk powder (Marvel) in phosphate buffered saline (PBS). Detection of scFv and VH protein sequences in the phage antibody fusion proteins was effected by soaking the filter for 1 hour with a 1/1000 dilution (in 2% milk powder) of a rabbit polyclonal antiserum raised against affinity purified, bacterially expressed scFv fragment (gift from G. Winter). After washing with PBS (3 x 5 minute washes), bound primary antibody was detected using an anti-rabbit antibody conjugated to horseradish peroxidase (Sigma, Fancy Road, Poole, Dorset, BH17 7NH, UK.) for 1 hour. The filter was washed in PBS/0.1% triton X-100 and developed with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.02% cobalt chloride, 0.03% hydrogen peroxide in PBS.

The results showed that with clones fdTVHD1.3 (from example 3 incorporating sequences coding for VH) and fdTscFvD1.3 (incorporating sequences coding for scFv) a protein of between 69,000 and 92,500 daltons is detected by the anti-Fv serum. This is the expected size for the fusion proteins constructed. This product is not observed in supernatants derived from fd-tet, fdT6Bst or fdTPs/Xh.

Example 3.

Insertion of Immunoglobulin VH Domain into Phage Antibody

The VH fragment from D1.3 was generated from the plasmid pSW1-VHD1.3-TAG1 (Ward, E.S. et al., 1989 supra.). Digestion of this plasmid with Pst1 and BstEII generates the fragment shown between positions 113 and 432 in figure 5. Cloning of this fragment into the Pst1 and BstEII sites of fdTPs/Bs gave rise to the construct fdTVHD1.3 which encodes a fusion protein with a complete VH domain inserted between the first and third amino acids of the mature gene III protein (amino acid two has been deleted).

The methods used were exactly as in example 2 except that the vector used was fdTPs/Bs digested with Pst1 and BstEII.

Example 4.

Analysis of Binding Specificity of Phage Antibodies

The binding of the various phage antibodies to the specific antigen, lysozyme, was analysed using ELISA techniques. Phage antibodies (e.g. fdTVHD1.3 and fdTsc/FvD1.3) were grown in E.coli and Phage antibody particles were precipitated with PEG as described in the materials and methods. Bound phage antibody particles were detected using polyclonal sheep serum raised against the closely related phage M13.

ELISA plates were prepared by coating 96 well plates (Falcon Microtest III flexible plate. Falcon: Becton Dickinson Labware, 1950 Williams Drive, Oxnard, California, 93030, USA.) with 200 ul of a solution of lysozyme (1mg/ml unless otherwise stated) in 50 mm NaHCO3 for 16-24 hours. Before use, this solution was removed, the plate rinsed several times in PBS and incubated with 200 ul of 2% milk powder/PBS for 1 hour. AFter rinsing several times with PBS, 100 ul of the test samples were added and incubated for 1 hour. Plates were washed (3 rinses in 0.05% Tween 20/PBS followed by 3 rinses in PBS alone). Bound phage antibodies were detected by adding 200 ul/well of a 1/1000 dilution of sheep anti-M13 polyclonal antiserum (gift from G. Winter, although an equivalent antibody can be readily made by one skilled in the art using standard methodologies) in 2% milk powder/PBS and incubating for 1 hour. After washing as above, plates were incubated with biotinylated anti-sheep antibody (Amersham International) for 30 minutes. Plates were washed as above, and incubated with streptavidin-horseradish peroxidase complex (Amersham International). After a final wash as above, 0.5 mg/ml ABTS substrate in citrate buffer was added (ABTS = 2'2'-azinobis (3-ethylbenzthiazoline sulphonic acid); citrate buffer = 50 mM citric acid, 50 mM tri-sodium citrate at a ratio of 54:46. Hydrogen peroxide was added to a final concentration of 0.003% and the plates incubated for 1 hour. The optical density at 405 nm was read in a Titertek multiskan plate reader.

Figure 6 shows the effect of varying the amount of phage antibody. 100 ul of various dilutions of PEG precipitated phage were applied and the amount expressed in terms of the original culture volume from which it was derived. Signals derived from both the scFv containing phage antibody (fdTscFvD1.3) and the VH containing phage antibody (fdTVHD1.3) and the VH containing phage antibody were higher than that derived from the phage antibody vector (fdTPs/Xh). The highest signal to noise ratio occurs using the equivalent of 1.3 mls of culture.

Figure 7 shows the results of coating the plates with varying concentrations of lysozyme or bovine serum albumin (BSA). The equivalent of 1 ml of the original phage antibody culture supernatant was used. The signals from supernatants derived from fdTscFvD1.3 were again higher than those derived from fdTPs/Xh when lysozyme coated wells were used. There was no significant difference between these two types of supernatant when the plates were coated with BSA. Broadly speaking the level of signal on the plates is proportional to the amount of lysozyme coated. These results demonstrate that the binding detected is specific for lysozyme as the antigen.

Example 5.

Construction of fd CAT 2

It would be useful to design vectors that enable the use of restriction enzymes that cut DNA infrequently, thus avoiding unwanted digestion of the antibody gene inserts within their coding sequence. Enzymes with an eight base recognition sequence are particularly useful in this respect, for example NotI and SfiI. Chaudhary et al (PNAS 87 p1066-1070, 1990) have identified a number of restriction sites which occur rarely in antibody variable genes. The applicant has designed and constructed a vector that utilises two of these sites, as an example of how this type of enzyme can be used. Essentially sites for the enzymes ApaLI and NotI were engineered into fdTPs/Xh to create fdCAT2.

The oligonucleotide:

5'ACT TTC AAC AGT TTC TGC GGC CGC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG TGC ACT GTG AGA ATA GAA 3'

was synthesised (supra fig 4 legend) and used to mutagenise fdTPs/Xh using an in vitro mutagenesis kit from Amersham International as described in example 1, to create fd-CAT2. The sequence of fd-CAT2 was checked around the site of manipulation by DNA sequencing. The final sequence around the insertion point within gene III is shown in figure 8.

N.B. fdCAT2 is also referred to herein by the alternative terminologies fd-tet-DOG1 and fdDOG1.

Example 6

Specific Binding of Phage-antibody (pAb) to Antigen

The binding of pAb D1.3 (fdTscFvD1.3 of example 2) to lysozyme was further analysed by ELISA.

Methods.

1. Phage growth.

Cultures of phage transduced bacteria were prepared in 10-100 mls 2 x TY medium with 15 µg/ml tetracycline and grown with shaking at 37°C for 16-24 hrs. Phage supernatant was prepared by centrifugation of the culture (10 min at 10,000 rpm, 8 x 50 ml rotor, Sorval RC-5B centrifuge). At this stage, the phage titre was 1 - 5 x 10_{10}/ml transducing units. The phage were precipitated by adding 1/5 volume 20% PEG 2.5 M NaCl, leaving for 1 hr at 4°C, and centrifuging (supra). The phage pellets were resuspended in 10 mM Tris-HCl, 1mM EDTA pH 8.0 to 1/100th of the original volume, and residual bacteria and aggregated phage removed by centrifugation for 2 min in a bench microcentrifuge.

ELISA

Plates were coated with antigen (1 mg/ml antigen) and blocked as described in example 4. 2 x 10_{10} phage transducing units were added to the antigen coated plates in phosphate buffered saline (PBS) containing 2% skimmed milk powder (MPBS). Plates were washed between each step

with three rinses of 0.5% Tween-20 in PBS followed by three rinses of PBS. Bound phage was developed by incubating with sheep anti-M13 antisera and detected with horseradish peroxidase (HRP) conjugated anti-goat serum (Sigma, Poole, Dorset, UK) which also detects sheep immunoglobulins and ABTS (2'2'-azinobis (3-ethylbenzthiazoline sulphonic acid). Readings were taken at 405 nm after a suitable period. The results (figure 9) show that the antibody bearing-phage had the same pattern of reactivity as the original D1.3 antibody (Harper, M., Lema, F., Boulot, G., and Poljak, F.J. (1987) Molec. Immunol. 24, 97-108), and bound to hen egg-white lysozyme, but not to turkey egg-white lysozyme, human lysozyme or bovine serum albumin. The specificity of the phage is particularly illustrated by the lack of binding to the turkey egg-white lysozyme that differs from hen egg-white lysozyme by only 7 amino acids.

Example 7.

Expression of Fab D1.3

The aim of this example was to demonstrate that the scFv format used in example 2 was only one way of displaying antibody fragments in the pAb system. A more commonly used antibody fragment is the Fab fragment (figure 1) and this example describes the construction of a pAb that expresses a Fab-like fragment on its surface and shows that it binds specifically to its antigen. The applicant chose to express the heavy chain of the antibody fragment consisting of the VH and CH1 domains from coding sequences within the pAb itself and to co-express the light chain in the bacterial host cell infected with the pAb. The VH and CH1 regions of anti-lysozyme antibody D1.3 were cloned in fd CAT2, and the corresponding light chain cloned in plasmid pUC19. The work of Skerra and Pluckthun (Science 240, p1038-1040 (1988) and Better et al 1988 supra; demonstrated that multimeric antigen binding fragments of the antibody molecule could be secreted into the periplasm of the bacterial cell in a functional form using suitable signal sequences. However, in these publications, special measures were described as being needed to recover the binding protein from the cell, for example Skerra and Pluckham needed to recover the Fv fragment from the periplasm by affinity chromatography. The present applicants have shown that it is possible to direct the binding molecule to the outside of the cell on a phage particle, a process that requires several events to occur: correct secretion and folding of the binding molecule; association of the chains of the binding molecule; correct assembly of the phage particle; and export of the intact phage particle from the cell.

Alternatively, it is possible however, to express the light chain from within the pAb genome by, for

example, cloning an expression cassette into a suitable place in the phage genome. Such a suitable place would be the intergenic region which houses the multicloning sites engineered into derivative of the related phage M13 (see, for example, Yanisch-Perron, C. et al., Gene 33, p103-119, (1985)).

The starting point for this example was the clone Fab D1.3 in pUC19, a map of which is shown in figure 10. The regions hybridising with the oligonucleotides KSJ6 and 7 below are shown underlined in fig 10. The sequence encoding the VH-CH1 region (defined at the 5' and 3' edges by the oligonucleotides KSJ6 and 7 below) was PCR amplified from Fab D1.3 in pUC19 using oligonucleotides KSJ 6 and 7, which retain the Pst I site at the 5' end and introduce a Xho I site at the 3' end, to facilitate cloning into fd CAT2. The sequences for the oligonucleotides KSJ6 and 7 are shown below. The underlined region of KSJ7 shows the portion hybridising with the sequence for D1.3.

KSJ6:5' AGG TGC AGC TGC AGG AGT CAG G 3'

KSJ7: 5' GGT GAC CTC GAG TGA AGA TTT GGG CTC AAC TTT C 3'

PCR conditions were as described in example II, except that thirty cycles of PCR amplification were performed with denaturation at 92°C for 45 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The template used was DNA from TG1 cells containing Fab D1.3 in pUC19 resuspended in water and boiled. The template DNA was prepared from the colonies by picking some colony material into 100µl of distilled H_2O and boiling for 10 mins. 1µl of this mixture was used in a 20µl PCR. This regime resulted in amplification of the expected fragment of approximately 600bp. This fragment was cut with Pst I and Xho I, purified from an agarose gel and ligated into Pst 1/Xho 1-cut fdCAT2. The PCR mixture was extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.) before digestion with Pstl and Xhol (New England Biolabs according to manufacturers recommendations. The fragment was resolved on 1% Tris-Acetate EDTA agarose gel (Sambrook et al. supra) and purified using Geneclean (BIO 101, Geneclean, La Jolla, San Diego, California, USA) according to manufacturers recommendations.

fd-CAT2 vector DNA was digested with Pst 1 and Xho 1 (New England BioLabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.).

75ng of Pst 1/Xho 1-digested vector DNA was ligated to 40ng of PCR-amplified Pst1 /Xho I-digested hEGF-R fragment in 12µl of ligation buffer (66mM TrisHCl (pH7.6), 5mM $MgCl_2$, 5mM dithiothreitol, (100µg/ml bovine serum albumin, 0.5mM ATP, 0.5mM Spermidine) and 40C units T4 DNA ligase (New England BioLabs) for 16 hours at 16°C.

Two µl of the ligation mixture was transformed into 200µl of competent E.coli MC1061 cells, plated on 2TY agar containing 15µg/ml tetracycline and incubated at 30°C for 20 hours. A portion of the ligation reaction mixture was transformed into E.coli MC1061 (Available from, for example Clontech Laboratories Inc, Palo Alto, California) and colonies identified by hybridisation with the oligonucleotide D1.3CDR3A as described in example 10. The presence of the VHCH1 gene fragment was likewise confirmed by PCR, using oligonucleotides KSJ6 and 7. A representative clone was called fd CAT2VHCH1 D1.3. The heavy chain was deleted from Fab D1.3 in pUC19 by Sph I cleavage of Fab D1.3 plasmid DNA. The pUC 19 2.7Kb fragment containing the light chain gene was purified from a TAE agarose gel, and 10ng of this DNA self-ligated and transformed into competent E.coli TG1. Cells were plated on 2TY agar containing ampicillin (100µg/ml) and incubated at 30°C overnight. The resulting colonies were used to make miniprep DNA (Sambrook et al. supra), and the absence of the heavy chain gene confirmed by digestion with Sph I and Hind III. A representative clone was called LCD1.3 DHC.

An overnight culture of fd CAT2VHCH1 D1.3 cells was microcentrifuged at 13,000Xg for 10 minutes and 50µl of the supernatant containing phage particles added to 50µl of an overnight culture of LCD1.3 DHC cells. The cells were incubated at 37°C for 10 minutes and plated on 2TY agar containing ampicillin (100µg/ml) and 15µg/ml tetracycline. Phage were prepared from some of the resulting colonies and assayed for their ability to bind lysozyme as described in example 6.

The results (Figure 11) showed that when the heavy and light chain Fab derivatives from the original antibody D1.3 were present, the pAb bound to lysozyme. pAb expressing the fd VHCH1 fragment did not bind to lysozyme unless grown in cells also expressing the light chain. This shows that a functional Fab fragment was produced by an association of the free light chain with VHCH1 fragment fused to gene III and expressed on the surface of the pAb.

Example 8

Isolation of Specific, Desired Phage from a Mixture of Vector Phage.

The applicant purified pAb (D1.3) (originally called fdTscFvD1.3 in example 2) from mixtures using antigen affinity columns. pAb (D1.3) was mixed with vector fd phage (see table 1) and approximately 10^{12} phage passed over a column of lysozyme-Sepharose (prepared from cyanogen bromide activated sepharose 4B (Pharmacia, Milton Keynes, Bucks, UK.) according to the manufacturers instructions. TG1 cells were infected with appropriate dilutions of the elutes and the colonies derived, were

analysed by probing with an oligonucleotide that detects only the pAb (D1.3) see Table 1 and Fig. 12. A thousand fold enrichment of pAb(D1.3) was seen with a single column pass. By growing the enriched phage and passing it down the column again, enrichments of up to a million fold were seen.

Enrichment was also demonstrated using purely immunological criteria. For example, 10^{12} phage (at a ratio of 1 pAb (D1.3) to 4 x 10^6 fdTPs/Bs) was subjected to two rounds of affinity selection, and then 26 colonies picked and grown overnight. The phage was then assayed for lysozyme binding by ELISA (as example 6). Five colonies yielded phage with lysozyme binding activities, see table 1, and these were shown to encode the scFv (D1.3) by PCR screening (example 13, using 30 cycles of 1 minute at 92°C, 1 minute at 60°C, 1 minute at 72°C using CDR3PCR1 and oligo 3 (fig. 4) as primers).

Thus very rare pAbs can be fished out of large populations, by using antigen to select and then screen the phage.

In this example, affinity chromatography of pAbs and oligonucleotide probing were carried out as described below.

Approximately 10^{12} phage particles in 1ml MPBS were loaded onto a 1 ml lysozyme-Sepharose affinity column which had been prewashed in MPBS. The column was washed in turn with 10 ml PBS; then 10 ml 50 mM Tris-HCl, 500 mM Nacl pH 7.5; then 10ml 50 mM Tris-HCl 500 mM NaCl pH 8.5; then 5 mls 50 mM Tris-HCl, 500 mM NaCl pH 9.5 (adjusted with triethylamine) and then eluted with 5 ml 100 mM triethylamine. The eluate was neutralised with 0.5 M sodium phosphate buffer pH 6.8 and the phage plated for analysis. For a second round of affinity chromatography, the first column eluate was plated to about 30,000 colonies per petri dish. After overnight growth, colonies were then scraped into 5 ml 2 x TY medium, and a 20 µl aliquot diluted into 10 ml fresh medium and grown overnight. The phage was PEG precipitated as described above, resuspended in 1 ml MPBS and loaded onto the column, washed and eluted as above.

Oligonucleotides sythesised:

CDR3PCR1 5'TGA GGA C(A or T) C(A or T) GC CGT CTA CTA CTG TGC 3'

40 pmole of oligonucleotide VH1FOR (Ward, E. S., et al (1989) Nature 341, 544-546), specific to pAb (D1.3) was phosphorylated with 100 µCi α-32P ATP, hybridised (1pmole/ml) to nitrocellulose filters at 67°C in 6 x saline sodium citrate (SSC) Sambrook et al., supra. buffer for 30 minutes and allowed to cool to room temperature for 30 mins, washed 3 x 1 min at 60°C in 0.1 x SSC.

Example 9

Construction of pAb Expressing Anti-hapten Activity

Oxazolone is a hapten that is commonly used for studying the details of the immune response. The anti-oxazalone antibody, NQ11 has been described previously (E. Gherardi, R. Pannell, C. Milstein, J. Immunol. Method 126 61-68). A plasmid containing the VH and VL gene of NQ11 was converted to a scFv form by inserting the BstEII/SacI fragment of scFvD1.3 myc (nucleotides 432-499 of Fig. 5) between the VH and VL genes to generate pscFvNQ11, the sequence of which is shown in fig. 13. This scFv was cloned into the Pst1/Xho1 site of FdTPs/Xh (as described earlier) to generate pAb NQ11 has an internal Pst1 site and so it was necessary to do a complete digest of pscFvNQ11 with Xho1 followed by a partial digest with Pst1).

The specific binding of pAb NQ11 was confirmed using ELISA. ELISA plates were coated at 37°C in 50 mM NaHCO3 at a protein concentration of 200 µg/ml. Plates were coated with either hen egg lysozyme (HEL), bovine serum albumin (BSA), or BSA conjugated to oxazolone (OX-BSA) (method of conjugation in Makela O.,, Kartinen M., Pelkonen J.L.T., Karjalainen K. (1978) J. Exp. Med.148 1644). Preparation of phage, binding to ELISA plates, washing and detection was as described in example 6. Samples were assayed in duplicate and the average absorbance after 10 minutes presented in figure 14.

This result demonstrates that the pAb NQ11 binds the correct antigen. Figure 14 also shows that pAb D1.3 and pAb NQ11 bind only to the antigen against which the original antibodies were raised.

Example 10

Enrichment of pAb D1.3 from Mixtures of Other pAb by Affinity Purification

3×10^{10} phage in 10 mls of PBSM at the ratios of pAb D1.3 to pAb NQ11 shown in table 2 were passed over a 1 ml lysozyme Sepharose column. Washing, elution and other methods were as described in example 8 unless otherwise stated. Eluates from the columns were used to infect TG1 cells which were then plated out. Colonies were probed with a probe which distinguishes pAb D1.3 from pAb NQ11. The sequence of this oligonucleotide (D1.3CDR3A) is:-

5'GTA GTC AAG CCT ATA ATC TCT CTC 3'

Table 2 presents the data from this experiment. An enrichment of almost 1000 fold was achieved in one round and an enrichment of over a million fold in two rounds of purification. This parallels the result described in example 8.

Example 11

Insertion of a Gene Encoding an Enzyme (Alkaline phosphatase) into fd-CAT2

As an example of the expression of a functional

enzyme on the bacteriophage surface, the applicants have chosen bacterial alkaline phosphatase, an enzyme that normally functions as a dimer (McCracken, S. and Meighen, E., J. Biol. Chem. 255, p2396-2404, (1980)). The oligonucleotides were designed to generate a PCR product with an Apa L1 site at the 5' end of phoA gene and a Not 1 site at its 3' end, thus facilitating cloning into fd-CAT 2 to create a gene III fusion protein. The oligonucleotides synthesised were:

phoA1:5' TAT TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG 3' and,

phoA2:5' ACA TGT ACA TGC GGC CGC TTT CAG CCC CAG AGC GGC TTT C3'

The sequence of the phoA gene is presented in Chang C. N. et al., Gene 44, p121-125 (1986). The plasmid amplified (pEK86) contains an alkaline phosphate gene which differs from the sequence of Chang et al, by a mutation which converts arginine to alamine at position 166.

The PCR reaction was carried out in 100µl of 10 mM Tris/HCl pH 8.3, containing 50 mM KCl, 5mMdNTP 2.5 mM $MgCl_2$, 0.01% gelatin, 0.25 units/µl of Taq polymerase (Cetus/Perkin Elmer) and 0.5µg/ml template. The template was the pEK86 plasmid (described by Chaidaroglou et al., Biochemistry 27 p8338-8343, 1988). The PCR was carried out in a Techne (Techne, Duxford, Cambridge, UK) PHC-2 dri-block using thirty cycles of 1 min at 92°C, 2 min at 50°C, 3 min at 72°C.

The resultant product was extracted with phenol:chloroform, precipitated with ethanol, and the pellet dissolved in 35µl water. Digestion with 0.3 units/µl of Apa L1 was carried out in 150µl volume according to manufacturers instructions for two hours at 37°C. After heat inactivation of the enzyme at 65°C , NaCl was added to a final concentration of 150mM and 0.4 units/µl Not1 enzyme added. After incubation for 2 hours at 37°C, the digest was extracted with phenol:chloroform and precipitated as above, before being dissolved in 30µl of water. The vector fd-CAT2 was sequentially digested with Apa L1 and Not1 according to the manufacturers instructions and treated with calf intestinal alkaline phosphatase as described in example 2. The sample was extracted three times with phenol:chloroform, precipitated with ethanol and dissolved in water. The ligations were performed with a final DNA concentration of 1-2ng/µl of both the cut fd-CAT2 and the digested PCR product. The ligations were transformed into competent TG1 cells and plated on 2xTY tet plates. Identification of clones containing the desired insert was by analytical PCR performed using the conditions and primers above, on boiled samples of the resulting colonies. The correct clone containing the phoA gene fused in frame to gene III was called fd-phoAla 166. The sequence at the junction

of the cloning region is given in figure 15.

Example 12

Measuring Enzyme Activity of Phage-enzyme

Overnight cultures of TG1 or KS272 (E.coli cells lacking phoA. Strauch K. L., and Beckwith J. PNAS 85 1576-1580, 1988) cells containing either fd-phoA1a 166 or fd-CAT2 were grown at 37°C in 2xTY with 15µg/ml tetracycline. Concentrated, PEG precipitated phage were prepared as described earlier. Enzyme assays (Malamy, M.H. and Horecker B.L., Biochemistry 3, p1893-1897, (1964)) were carried out at 24°C in a final concentration of 1M Tris/HCl pH 8.0, 1mM 4-nitrophenyl phosphate (Sigma), 1mM MgCl2. 100µl of a two times concentrate of this reaction mixture was mixed with 100µl of the test sample in a 96 well plate. Absorbance readings were taken every minute for 30 minutes at a wavelength of 405nm in a Titretek Mk 2 plate reader. Initial reaction rates were calculated from the rate of change of absorbance using a molar absorbance of 17000 l/mol/cm.

Standard curves (amount of enzyme vs. rate of change of absorbance) were prepared using dilutions of purified bacterial alkaline phosphatase (Sigma type III) in 10mM Tris/HCl pH 8.0, 1mM EDTA. The number of enzyme molecules in the phage samples were estimated from the actual rates of change of absorbance of the phage samples and comparison to this standard curve.

The results in Table 3 show that alkaline phosphatase activity was detected in PEG precipitated material in the sample containing fd-phoA1a166 but not fd-CAT2. Furthermore, the level of activity was consistent with the expected number of 1-2 dimer molecules of enzyme per phage. The level of enzyme activity detected was not dependent on the host used for growth. In particular, fd-phoA1a166 grown on phoA minus hosts showed alkaline phosphatase activity.

Therefore, the phage expressed active alkaline phosphatase enzyme, from the phoA-gene III fusion, on the phage surface.

Example 13

Insertion of Binding Molecules into Alternative Sites in the Phage

The availability of an alternative site in the phage for the insertion of binding molecules would open up the possibility of more easily expressing more than one binding molecule e.g. an antibody fragment in a single pAb. This may be used to generate single or multiple binding specificities. The presence of two distinct binding activities on a single molecule will greatly increase the utility and specificity of this molecule. It may be useful in the binding of viruses with a high mutational rate such as human immunodeficiency virus. In addition, it may be used to bring antigens into close

proximity (e.g. drug targetting or cell fusion) or it may act as a "molecular clamp" in chemical, immunological or enzymatic processes.

The vector fd-tet and the derivatives described here, have a single BamH1 site in gene 3. This has previously been used for the expression of peptide fragments on the surface of filamentous bacteriophage (Smith GP. (1985) Science 228 p1315-1317 and de la Cruz et al. (1988) J Biol. Chem. 263 p4318-4322). This provides a potential alternative site for the insertion of antibody fragments.

DNA fragments encoding scFv's from D1.3 or NQ11 were generated by PCR using the primers shown below. These primers were designed to generate a fragment with BamH1 sites near both the terminii, to enable cloning into the BamH1 site of gene3 (see figure 16(1)). The oligonucleotides used, also ensure that the resulting PCR product lacks Pst1 and Xhol restriction sites normally used for manipulating the scFv's (see figure 16(1)). This will facilitate subsequent manipulation of a second antibody fragment in the usual way at the N terminus of gene 3. The oligonucleotides used were:-

G3Bam1 5'TTT AAT GAG GAT CCA CAG GTG CAG CTG CAA GAG 3'

G3Bam2 5'AAC GAA TGG ATC CCG TTT GAT CTC AAG CTT 3'.

Preparation of vector and PCR insert

The PCR reaction was carried out in an 80 µl reaction as described in example 11 using 1ng/µl of template and 0.25U/µl of Taq polymerase and a cycle regime of 94°C for 1 minute, 60°C for 1 minute and 70°C for 2 minutes over 30 cycles. The template was either pscFvNQ11 (example 9) or scFvD1.3 myc (example 2). Reaction products were extracted with phenol:chloroform, precipitated, dissolved in water and digested with BamH1 according to manufacturers instructions. The digest was re-extracted with phenol: chloroform, precipitated and dissolved in water.

The vector fdTPs/Xh was cleaved with BamH1 and treated with calf intestinal phosphatase and purified as described in example 2. Ligations were set up at a vector concentration of approximately 6ng/µl and a PCR insert concentration of approximately 3ng/µl. These were ligated for 2.5 hours at room temperature before transforming into competent TG1 cells and plating on TY tet plates. The resultant colonies were probed as described in example 8. DNA was prepared from a number of colonies and the correct orientation and insert size confirmed by restriction digestion with Hind III in isolation or in combination with BamH1. (One Hind III site is contributed by one of the primers and the other by the vector).

Two clones containing a D1.3 insert (fdTBam1) and fdTBam2) and one containing an NQ11 insert (NQ11Bam1)

were grown up and phage prepared as described earlier. ELISAs were carried out as described in example 6. No specific signal was found for any of these clones suggesting that the natural BamH1 site is not a suitable site for insertion of a functional antibody (results not shown).

It may be possible to clone into alternative sites to retain binding activity. The peptide repeats present in gene III may provide such a site (figure 16 blocks A and B). This can be done by inserting a BamH1 site and using the PCR product described above. To facilitate this, the natural BamH1 site was removed by mutagenesis with the oligonucleotide G3mutδBam shown below (using an in vitro mutagenesis kit (Amersham International)):-

G3mutδBam 5' CA AAC GAA TGG GTC CTC CTC ATT A 3'

The underlined residue replaces an A residue, thereby removing the BamH1 site. DNA was prepared from a number of clones and several mutants lacking BamH1 sites identified by restriction digestion.

The oligonucleotide G3 Bamlink was designed to introduce a BamH1 site at a number of possible sites within the peptide linker sites A and B, see figure 16(2). The sequence of the linker is:

Bamlink 5'CC (G or A) CC ACC CTC GGA TCC (G or A) CC ACC CTC 3'

Its relationship to the peptide repeats in gene III is shown in figure 16.

Example 14

PCR Assembly of Mouse VH and VL Kappa (VLK) Repertoires for Phage Display

The principle is illustrated in figure 17. Details are provided in sections A to F below but the broad outline is first discussed.

1. cDNA is prepared from spleen RNA from an appropriate mouse and the VH and VLK repertories individually amplified. Separately, primers reverse and complementary to VH1FOR-2 (domain 1) and VLK2BACK (domain 2) are used to amplify an existing scFv-containing DNA by PCR. (The term FOR refers to e.g. a primer for amplification of sequences on the sense strand resulting in antisense coding sequences. The term BACK refers to e.g. a primer for amplification of sequences on the antisense strand resulting in sense coding sequences). This generates a 'linker' molecule encoding the linker with the amino acid sequence (1 letter code) $(GGGGS)_3$ which overlaps the two primary (VH and VLK) PCR products.
2. The separate amplified VH, VLK and linker sequences now have to be assembled into a continuous DNA molecule by use of an 'assembly' PCR. In the secondary 'assembly' PCR, the VH, VLK and linker bands are combined and assembled by virtue of the

above referred to overlaps. This generates an assembled DNA fragment that will direct the expression of VH and one VLK domain. The specific VH/VLK combination is derived randomly from the separate VH and VLK repertoires referred to above.

The assembly PCR is carried out in two stages. Firstly, 7 rounds of cycling with just the three bands present in the PCR, followed by a further 20 rounds in the presence of the flanking primers VH1BACK (referring to domain 1 of VH) and VLKFOR. The nucleotide sequences for these oligonucleotide primers are provided under the section entitled 'Primer Sequences' below. This two stage process, avoids the potential problem of preferential amplification of the first combinations to be assembled.

For cloning into the phage system, the assembled repertoires must be 'tagged' with the appropriate restriction sites. In the example provided below this is illustrated by providing an ApaLI restriction site at the VH end of the continuous DNA molecule and a Not 1 site at the VLK end of the molecule. This is carried out by a third stage PCR using tagged primers. The nucleotide sequences for these oligonucleotide primers are also provided under the section entitled 'Primer Sequences' below. There are however, 4 possible kappa light chain sequences (whereas a single consensus heavy chain sequence can be used). Therefore 4 oligonucleotide primer sequences are provided for VLK.

For this third stage PCR, sets of primers which create the new restriction site and have a further 10 nucleotides on the 5' side of the restriction site have been used. However, long tags may give better cutting, in which case 15-20 nucleotide overhangs could be used.

Scrupulously clean procedures must be used at all times to avoid contamination during PCR. Negative controls containing no DNA must always be included to monitor for contamination. Gel boxes must be depurinated. A dedicated Geneclean kit (B10 101, Geneclean, La Jolla, San Diego, California, USA) can be used according to manufacturers instructions to extract DNA from an agarose gel. The beads, NaI and the NEW wash should be aliquoted.

All enzymes were obtained from CP Laboratories, P.O. Box 22, Bishop's Stortford, Herts CM20 3DH and the manufacturers recommended and supplied buffers were used unless otherwise stated.

A. RNA Preparation

RNA can be prepared using may procedures well known to those skilled in the art. As an example, the following protocol (Triton X-100 lysis, phenol/SDS RNase inactivation) gives excellent results with spleen and hybridoma cells (the addition of VRC (veronal ribosyl

complex) as an RNase inhibitor is necessary for spleen cells). Guanidinium isothiocyanate/CsCl procedures (yielding total cellular RNA) also give good results but are more time-consuming.

1. Harvest 1 to 5 x 10^7 cells by centrifugation in a bench tope centrifuge at 800xg for 10 minutes at 4°C. Resuspend gently in 50ml of cold PBS buffer. Centrifuge the cells again at 800xg for 10 minutes at 4°C, and discard supernatant.
2. On ice, add 1 ml ice-cold lysis buffer to the pellet and resuspend it with a 1ml Gilson pepette by gently pepetting up and down. Leave on ice for 5 minutes.
3. After lysis, remove cell debris by centrifuging at 1300 rpm for 5 minutes in a microfuge at 4°C, in precooled tubes.
4. Transfer 0.5 ml of the supernatant to each of two eppendorfs containing 60µl 10% (w/v) SDS and 250 µl phenol (previously equilibrated with 100 mM Tris-HCl pH 8.0). Vortex hard for 2 minutes, then microfuge (13000 rpm) for five minutes at room temperature. Transfer the upper, aqueous, phase to a fresh tube.
5. Re-extract the aqueous upper phase five times with 0.5 ml of phenol.
6. Precipitate with 1/10 volume 3M sodium acetate and 2.5 volumes ethanol at 20°C overnight or dry ice-isopropanol for 30 minutes.
7. Wash the RNA pellet and resuspended in 50 µl to check concentration by OD260 and check 2 µg on a 1% agarose gel. 40µg of RNA was obtained from spleen cells derived from mice.

Lysis buffer is [10mM Tris-HCl pH 7.4, 1mM MgCl2, 150mM NaCl, 10mM VRC (New England Biolabs), 0.5% (w/v) Triton X-100], prepared fresh.

Lysis buffer is [10mM Tris-HCl pH 7.4, 1mM $MgCl_2$, 150mM NaCl, 10mM VRC (New England Biolabs), 0.5% (w/v) Triton X-100], prepared fresh.

B. cDNA Preparation

cDNA can be prepared using many procedures well known to those skilled in the art. As an example, the following protocol can be used:

1. Set up the following reverse transcription mix:

	µl
H_2O (DEPC-treated)	20
5mM dNTP	10
10 x first strand buffer	10
0.1M DTT	10
FOR primer(s) (10 pmol/µl)	2 (each) (see below)
RNasin (Promega; 40 U/µl)	4

NB

i) DEPC is diethylpyrocarbonate, the function of which is to inactivate any enzymes that could degrade DNA

or RNA

ii) dNTP is deoxynucleotide triphosphate

iii) DTT is dithiothreitol the function of which is as an antioxidant to create the reducing environment necessary for enzyme function.

iv) RNasin is a ribonuclease inhibitor obtained from Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA.

2. Dilute 10 µg RNA to 40 µl final volume with DEPC-treated water. Heat at 65°C for 3 minutes and hold on ice for one minute (to remove secondary structure).

3. Add to the RNA the reverse transcription mix (58 µl) and 4 µl of the cloned reverse transcriptase 'Super RT' (Anglian Biotech Ltd., Whitehall House, Whitehall Road, Colchester, Essex) and incubate at 42°C for one hour.

4. Boil the reaction mix for three minutes, cool on ice for one minute and then spin in a microfuge to pellet debris. Transfer the supernatant to a new tube.

10 x first strand buffer is [1.4M KCl, 0.5M Tris-HCl pH 8.1 at 42°C 80mM $MgCl_2$].

The primers anneal to the 3' end. Examples of kappa light chain primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (provided under 'Primer Sequences' below) and examples of heavy chain primers are MIGG1, 2 (CTG GAC AGG GAT CCA GAG TTC CA) and MIGG3 (CTG GAC AGG GCT CCA TAG TTC CA) which anneal to CH1.

Alternatively, any primer that binds to the 3' end of the variable regions VH, VLK, VL, or to the constant regions CH1, CK or CL can be used.

C. Primary PCRs

For each PCR and negative control, the following reactions are set up (e.g. one reaction for each of the four VLKs and four VH PCRs). In the following, the Vent DNA polymerase sold by (C.P. Laboratories Ltd (New England Biolabs) address given above) was used. The buffers are as provided by C.P. Laboratories.

	µl
H_2O	32.5
10 x Vent buffer	5
20 x Vent BSA	2.5
5mM dNTPs	1.5
FOR primer 10 pmol/µl)	2.5
BACK primer 10pmol/µl	2.5

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. For VH, the FOR primer is VH1FOR-2 and the BACK primer is VH1BACK. For VLK the FOR primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (for the four respective kappa light chains) and the BACK

primer is VK2BACK. Only one kappa light chain BACK primer is necessary, because binding is to a nucleotide sequence common to the four kappa light chains.

UV this mix 5 minutes. Add 2.5 µl cDNA preparation (from B above), 2 drops paraffin oil (Sigma Chemicals, Poole, Dorset, UK). Place on a cycling heating block, e.g. PHC-2 manufactured by Techne Ltd. Duxford UK, pre-set at 94°C. Add 1µl Vent DNA polymerase under the paraffin. Amplify using 25 cycles of 94°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

Purify on a 2% lmp (low melting point agarose/TAE (tris-acetate EDTA)gel and extract the DNA to 20 µl H_2O per original PCR using a Geneclean kit (see earlier) in accordance with the manufacturers instructions.

D. Preparation of linker

Set up in bulk (e.g. 10 times)

	µl
H_2O	34.3
10 x Vent buffer	5
20 x Vent BSA	2.5
5mM dNTPs	2
LINKFOR primer 10 pmol/µl)	2.5
LINKBACK primer 10pmol/µl	2.5
DNA from fcFv D1.3 (example 2)	1
Vent enzyme	0.2

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is LINKFOR and the BACK primer is LINKBACK. Cover with paraffin and place on the cycling heating block (see above) at 94°C. Amplify using 25 cycles of 94°C 1 min, 65°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

Purify on 2% lmp/TAE gel (using a loading dye without bromophenol blue as a 93bp fragment is desired) and elute with SPIN-X column (Costar Limited, 205 Broadway, Cambridge, Ma. USA.,) and precipitation. Take up in 5 µl H_2O per PCR reaction.

E. Assembly PCRs

A quarter of each PCR reaction product (5µl) is used for each assembly. The total volume is 25ul.

For each of the four VLK primers, the following are set up:

H_2O	4.95
10 x Vent buffer	2.5
20 x Vent BSA	1.25
5mM dNTPs	0.8

UV irradiate this mix for 5 min. Add 5ul each of Vh and VK band from the primary PCRs and 1.5 ul of linker as isolated from the preparative gels and extracted using the Geneclean kit as described in C and D above. Cover

with paraffin. Place on the cycling heating block preset at 94°C. Add 1µl Vent under the paraffin. Amplify using 7 cycles of 94°C 2 min, 72°C 4 min. Then return the temperature to 94°C.

Add 1.5µl each of VH1BACK and the appropriate VKFOR primers MJK1FONX, MJK2FONX. MJK4FONX or MJK5FONX (10 pmol/µl) at 94°C. The primers should have been UV-treated as above. Amplify using 20 cycles of 94°C 1.5 min, 72°C 2.5 min. Post-treat at 60°C for 5 min. Purify on 2% lmp/TAE gel and extract the DNA to 20µl H_2O per assembly PCR using a Geneclean kit (see earlier) in accordance with the manufacturers instructions.

F. Adding Restriction Sites

For each assembly and control set up:

	µl
H_2O	36.5
10 x Taq buffer	5
5mM dNTPs	2
FOR primer (10 pmol/µl)	2.5
BACK primer (10 pmol/µl)	2.5
Assembly product	1

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is any of JK1NOT10, JK2NOT10, JK4NOT10 or JK5NOT10 (for the four respective kappa light chains) for putting a NotI restriction site at the VLK end. The BACK primer is HBKAPA10 for putting an ApaL1 restriction site at the VH end.

Cover with paraffin and place on the cycling heating block preset at 94°C. Add 0.5 µl Cetus Taq DNA polymerase (Cetus/perkin-Elmer, Beaconsfield, Bucks, UK) under the paraffin. Amplification is carried out using 11 to 15 rounds of cycling (depends on efficiency) at 94°C 1 min, 55°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

10 x Taq buffer is [0.1M Tris-HCl pH 8.3 at 25°C, 0.5M KCl, 15mM $MgCl_2$, 1mg/ml gelatin].

G. Work-up

Purify once with $CHCl_3$/IAA (isoamylalcohol), once with phenol, once with $CHCl_3$/IAA and back-extract everything to ensure minimal losses. Precipitate and wash twice in 70% EtOH. Dissolve in 70ul H_2O.

Digest overnight at 37°C with NotI:

	µl
DNA (joined seq)	70
NEB NotI buffer x 10	10
NEB BSA x 10	10
NotI (10 U/µl)	10

The DNA (joined sequence) above refers to the assembled DNA sequence comprising in the 5' to 3' direction

ApaL1 restriction site
VH sequence

Linker sequence
VLK sequence
Not 1 restriction site.

The VLK sequence may be any one of four possible kappa chain sequences.

The enzymes Not 1 above, ApaL1 below and the buffers NEB Not 1, NEB BSA above and the NEB buffer 4 (below) are obtainable from CP Laboratories, New England Biolabs mentioned above.

Re-precipitate, take up in 80µl H_2O. Add to this 10µl NEB buffer 4 and 10µl Apal 1.

Add the enzyme ApaL1 in aliquots throughout the day, as it has a short half-life at 37°C.

Purify on 2% 1mp/TAE gel and extract the DNA using a Geneclean kit, in accordance with the manufacturers instructions. Redigest if desired.

H. Final DNA product

The final DNA product is an approximate 700 bp fragment with Apa L1 and Notl compatible ends consisting of randomly associated heavy and light chain sequences linked by a linker. A typical molecule of this type is the scFvDl.3 molecule incorporated into fdscFvDl.3 described in example 3. These molecules can then be ligated into suitable fd derived vectors, e.g. fdCAT2 (example 5), using standard techniques.

Primer sequences

Primary PCR oligos (restrictions sites underlined):

VH1FOR-2 TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC
VH1BACK AGG TSM ARC TGC AGS AGT CWG G
MJK1FONX CCG TTT GAT TTC CAG CTT GGT GCC
MJK2FONX CCG TTT TAT TTC CAG CTT GGT CCC
MJK4FONX CCG TTT TAT TTC CAA CTT TGT CCC
MJK5FONX CCG TTT CAG CTC CAG CTT GGT CCC
VK2BACK GAC ATT GAG CTC ACC CAG TCT CCA

Ambiguity codes M = A or C, R = A or G, S = G or C, W = A or T

PCR oligos to make linker:

LINKFOR TGG AGA CTC GGT GAG CTC AAT GTC
LINKBACK GGG ACC ACG GTC ACC GTC TCC TCA

For adding restriction sites:

HBKAPA10 CAT GAC CAC AGT GCA CAG GTS MAR CTG CAG SAG TCW GG
JKINOT10 GAG TCA TTC TGC GGC CGC CCG TTT GAT TTC CAG CTT GGT GCC
JK2NOT10 GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT GGT CCC
JK4NOT10 GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAA CTT TGT CCC
JK5NOT10 GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT GGT CCC

Example 15

Insertion of the Extracellular Domain of a Human Receptor for Platelet Derived Growth Factor (PDGF) soform BB into fd CAT2

A gene fragment encoding the extracellular domain of the human receptor for platelet derived growth factor isoform BB (h-PDGFB-R) was isolated by amplification, using the polymerase chain reaction, of plasmid RP41, (from the American Type Culture collection, Cat. No.50735), a cDNA clone encoding amino-acids 43 to 925 of the PDGF-B receptor (Gronwald, R.G.K. et al PNAS 85 p3435-3439 (1988)). Amino acids 1 to 32 of h-PDGFB-R constitute the signal peptide. The oligonucleotide primers were designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primer RPDGF3 for the N-terminal region also included bases encoding amino acids 33 to 42 of the h-PDGFB-R protein (corresponding to the first ten amino acids from the N-terminus of the mature protein) to enable expression of the complete extracellular domain. The primers also incorporate a unique ApaLl site at the N-terminal end of the fragment and a unique Xhol site at the C terminal end to facilitate cloning into the vector fdCAT2. The sequence of the primers is:

RPDGF3 5' CAC AGT GCA CTG GTC GTC ACA CCC CCG GGG CCA GAG CTT GTC CTC AAT GTC TCC AGC ACC TTC GTT CTG 3'
RPDGF2 5' GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl Acids Research 18 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH7.3 at 70°C, 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dCTP, dGTP and dTTP, 500ng/ml RP41 DNA, 1µM each primer and 50 units/ml Taq polymerase (Cetus/Perkin Elmer, Beaconsfield, Bucks, U.K.). Thirty cycles of PCR were performed with denaturation at 92°C for 1 min, annealing at 60°C for 1min and extension at 72°C for 1.5 min. This reaction resulted in amplification of a fragment of ca. 1500bp as expected.

fdCAT2 vector DNA (see example 5) was digested with ApaLl and Xhol (New England Biolabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al, supra). Cloning of amplified RP41 DNA into this vector and identification of the desired clones was performed essentially as in example 7 except that digestion of the PCR product was with ApaLl and Xho 1. Colonies containing h-PDGFB-R DNA were identified by probing with 32p labelled RPDGF2 and the presence of an insert in hybridising colonies was confirmed by analytical PCR using RPDGF3 and RPDGF2 using the

conditions described in example 7.

Example 16
Binding of 125I-PDGF-BB to the Extracellular Domain of the Human Receptor for Platelet Derived Growth Factor Isoform BB Displayed on the Surface of fd Phage. Measured using an Immunoprecipitation Assay.

Phage particles, expressing the extracellular domain of the human platelet derived growth factor isoform BB receptor (fd h-PDGFB-R), were prepared by growing E.coli MC1061 cells transformed with fd h-PDGFB-R in 50ml of 2xTY medium with 15ug/ml tetracycline for 16 to 20 hours. Phage particles were concentrated using polyethylene glycol as described in example 6 and resuspended in PDGF binding buffer (25mM HEPES, pH7.4, o.15mM NaCl, 1mM magnesium chloride, 0.25% BSA) to 1/33rd of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 min in a mocrocentrifuge. Immunoblots using an antiserum raised against gene III protein (Prof. I. Rashed, Konstanz, Germany) show the presence in such phage preparations of a geneIII-h-PDGFB-R protein of molecular mass 125000 corresponding to a fusion between h-PDGFB-R external domain (55000 daltons) and geneIII (apparent molecular mass 70000 on SDS-polyacrylamide gel).

Duplicate samples of 35µl concentrated phage were incubated with ^{125}I-PDGF-BB (78.7fmol, 70nCi, 882Ci/mmol; Amersham International plc, Amersham, Bucks) for 1 hour at 37°C. Controls were included in which fdTPs/Bs vector phage (figure 4) or no phage replaced fd h-BDGFB-R phage. After this incubation, 10ul of sheep anti-M13 polyclonal antiserum (a gift from M. Hobart) was added and incubation continued for 30 min at 20°C. To each sample, 40ul (20ul packed volume) of protein G Sepharose Fast Flow (Pharmacia, Milton Keynes) equilibrated in PDGF binding buffer was added. Incubation was continued for 30 min at 20°C with mixing by end over end inversion on a rotating mixer. The affinity matrix was spun down in a microcentrifuge for 2 min and the supernatant removed by aspiration. Non-specifically bound ^{125}I-PDGF-BB was removed by resuspension of the pellet in 0.5ml PDGF binding buffer, mixing by rotation for 5 min, centrifugation and aspiration of the supernatant, followed by two further washes with 0.5ml 0.1% BSA, 0.2% Triton-X-100. The pellet finally obtained was resuspended in 100ul PDGF binding buffer and counted in a Packard gamma counter. For displacement studies, unlabelled PDGF-BB (Amersham International) was added to the stated concentration for the incubation of ^{125}I-PDGF-BB with phage.

^{125}I-PDGF-BB bound to the fd h-PDGFB-R phage and was immunoprecipitated in this assay. Specific binding to

receptor phage was 3.5 to 4 times higher than the non-specific binding with vector phage fdTPs/Bs or no phage (fig. 19). This binding of ^{125}I-PDGF-BB could be displaced by the inclusion of unlabelled PDGF-BB in the incubation with phage at 37°C (fig. 20). At 50nM, unlabelled PDGF-BB the binding of ^{125}I-PDGF-BB was reduced to the same level as the fdTPs/Bs and no phage control. Figure 21 shows the same data, but with the non-specific binding to vector deducted.

These results indicate that a specific saturable site for ^{125}I-PDGF-BB is expressed on fd phage containing cloned h-PDGFB-R DNA. Thus, the phage can display the functional extracellular domain of a cell surface receptor.

Example 17, Construction of Phagemid Containing GeneIII fused with the Coding Sequence for a Binding Molecule

It would be useful to improve the transfection efficiency of the phage-binding molecule system and also to have the possibility of displaying different numbers and specificities of binding molecules on the surface of the same bacteriophage. The applicants have devised a method that achieves both aims.

The approach is derived from the phagemid system based on pUC119 [Vieira, J and Messing, J. (1987) Methods Enzymol. 153:3]. In brief, gene III from fd-CAT2 (example 5) and gene III scFv fusion from fd-CAT2 scFv D1.3 (example 2) were cloned downstream of the lac promoter in separate samples of pUC119, in order that the inserted gene III and gene III fusion could be 'rescued' by M13M07 helper phage [Vieira, J and Messing, J. et supra.] prepared according to Sambrootz et al. 1989 supra. The majority of rescued phage would be expected to contain a genome derived from the pUC119 plasmid that contains the binding molecule-gene III fusion and should express varying numbers of the binding molecule on the surface up to the normal maximum of 3-5 molecules of gene III of the surface of wild type phage. The system has been exemplified below using an antibody as the binding molecule.

An fdCAT2 containing the single chain Fv form of the D1.3 antilysozyme antibody was formed by digesting fdTscFvD1.3 (example 2) with PstI and XhoI, purifying the fragment containing the scFv fragment and ligating this into PstI and XhoI digested fdCAT2. The appropriate clone, called fdCAT2 scFvD1.3 was selected after plating onto 2xTY tetracycline (15µg/ml) and confirmed by restriction enzyme and sequence analysis.

Gene III from fd-CAT2 (example 5) and the gene III scFv fusion from fd-CAT2 scFvD1.3 was PCR-amplified using the primers A and B shown below:

Primer A: TGC GAA GCT TTG GAG CCT TTT TTT TTG GAG ATT TTC AAC G

Primer B: CAG TGA ATT CCT ATT AAG ACT CCT TAT TAC GCA GTA TGT TAG C

Primer A anneals to the 5' end of gene III including the ribosome binding site is located and incorporates a Hind III site. Primer B anneals to the 3' end of gene III at the C-terminus and incorporates two UAA stop codons and an EcoR1 site. 100 ng of fd-CAT2 and fd-CAT2 scFv D1.3 DNA was used as templates for PCR-amplification in a total reaction volume of 50µl as described in example 7, except that 20 cycles of amplification were performed: 94°C 1 minute, 50°C 1 minute, 72°C 3 minutes. This resulted in amplification of the expected 1.2Kb fragment from fd-CAT2 and a 1.8Kb fragment from fd-CAT2 scFv D1.3.

The PCR fragments were digested with EcoR1 and Hind III, gel-purified and ligated into Eco-R1- and Hind III-cut and dephosphorylated pUC119 DNA and transformed into E.coli TG1 using standard techniques (Sambrook et al., et supra). Transformed cells were plated on SOB agar (Sambrook et al. 1989 supra) containing 100µg/ml ampicillin and 2% glucose. The resulting clones were called pCAT-3 (derived from fd-CAT2) and pCAT-3 scFv D1.3 (derived from fd-CAT2 scFv D1.3).

Example 18, Rescue of Anti-Lysozyme Antibody Specificity from pCAT-3 scFv D1.3 by M13KO7

Single pCAT-3 and pCAT-3 scFv D1.3 colonies were picked into 1.5ml 2TY containing 100µg/ml ampicillin and 2% glucose, and grown 6 hrs at 30°C. 30µl of these stationary cells were added to 6mls 2YT containing 100µg/ml ampicillin and 2% glucose in 50ml polypropylene tubes (Falcon, Becton Dickinson Labware, 1950 Williams Drive, Oxnard, CA. USA) and grown for 1.5 hrs at 30°C at 380rpm in a New Brunswick Orbital Shaker (New Brunswick Scientific Ltd., Edison House 163 Dixons Hill road, North Mimms, Hatfield, UK). Cells were pelleted by centrifugation at 5,000g for 25 minutes and the tubes drained on tissue paper. The cell pellets were then suspended in 6mls 2TY containing 1.25×10^9 p.f.u. ml^{-1} M13KO7 bacteriophage added. The mixture was left on ice for 5 minutes followed by growth at 35°C for 45 minutes at 450rpm. A cocktail was then added containing 4µl 100µg/ml ampicillin, 0.5µl 0.1M IPTG and 50µl 10mg/ml kanamycin, and the cultures grown overnight at 35°C, 450rpm.

The following day the cultures were centrifuged and phage particles PEG precipitated as described in example 6. Phage pellets were resuspended in 100µl TE (tris-EDTA see example 6) and phage titred on E.coli TG1. Aliquots of infected cells were plated on 2TY containing either 100µg/ml ampicillin to select for pUC119 phage particles, or 50µg/ml kanamycin to select for the M13 KO7 helper phage. Plates were incubated overnight at 37°C and

antibiotic-resistant colonies counted:

DNA	amp^R	kan^R
pCAT-3	$1.8x10^{11}$ colonies	$1.2x10^9$ colonies
pCAT-3scFv D1.3	$2.4x10^{11}$ colonies	$2.0x10^9$ colonies

This shows that the amp^R phagemid particles are infective and present in the rescued phage population at a 100-fold excess over kan^R M13KO7 helper phage.

Phage were assayed for anti-lysozyme activity by ELISA as described in example 6, with the following modifications:

1) ELISA plates were blocked for 3 hrs with 2% Marvel/PBS.

2) 50µl phage, 400µl 1xPBS and 50µl 20% Marvel were mixed end over end for 20 minutes at room temperature before adding 150µl per well.

3) Phage were left to bind for 2 hours at room temperature.

4) All washes post phage binding were:
2 quick rinses PBS/0.5% Tween 20
3x2 minute washes PBS/0.5% Tween 20
2 quick rinses PBS no detergent
3x2 minute washes PBS no detergent

The result of this ELISA is shown in figure 22, which shows that the antibody specificity can indeed be rescued efficiently.

It is considered a truism of bacterial genetics that when mutant and wild-type proteins are co-expressed in the same cell, the wild-type proteins are co-expressed in same cell, the wild-type protein is used preferentially. This is analogous to the above situation wherein mutant (i.e. antibody fusion) and wild-type gene III proteins (from M13KO7) are competing for assembly as part of the pUC119 phagemid particle. It is therefore envisaged that the majority of the resulting pUC 119 phage particles will have fewer gene III-antibody fusion molecules on their surface than is the case for purely phage system described for instance in example 2. Such phagemid antibodies are therefore likely to bind antigen with a lower avidity than fd phage antibodies with three or more copies of the antibody fusion on their surfaces (there is no wild-type gene III, in the system described, for instance, in example 2), and provide a route to production of phage particles with different numbers of the same binding molecule (and hence different acidities for the ligand/antigen) or multiple different binding specificities on their surface, by using helper phage such as M13KO7 to rescue cells expressing two or more gene III-antibody fusions.

It is also possible to derive helper phage that do not encode a functional gene III in their genomes (by for example deleting the gene III sequence or a portion of it or by incorporating an amber mutation within the gene).

These defective phages will only grow on appropriate cells (for example that provide functional gene III in trans, or contain an amber supressor gene), but when used to rescue phage antibodies, will only incorporate the gene III antibody fusion encoded by the phagemid into the released phage particle.

Example 19. Transformation Efficiency of pCAT-3 and pCAT-3 scFv D1.3 phagemids

pUC 19, pCAT-3 and pCAT-3 scFv D1.3 plasmid DNAs, and fdCAT-2 phage DNA was prepared, and used to transform E.coli TG1, pCAT-3 and pCAT-3 scFv D1.3 transformations were plated on SOB agar containing 100µg/ml ampicillin and 2% glucose, and incubated overnight at 30°C. fdCAT-2 transformations were plated on TY agar containing 15µg/ml tetracycline and incubated overnight at 37°C. Transformation efficiencies are expressed as colonies per µg of input DNA.

DNA	Transformation efficiency
pUC 19	1.10^9
pCAT-3	1.10^8
pCAT-3scFv D1.3	1.10^8
fd CAT-2	8.10^5

As expected, transformation of the phagemid vector is approximately 100-fold more efficient that the parental fdCAT-2 vector. Furthermore, the presence of a scFv antibody fragment does not compromise efficiency. This improvement in transformation efficiency is practically useful in the generation of phage antibodies libraries that have large repertoires of different binding specificities.

Example 20

PCR Assembly of a Single Chain Fv Library from an Immunised Mouse

To demonstrate the utility of phage for the selection of antibodies from repertoires, the first requirement is to be able to prepare a diverse, representative library of the antibody repertoire of an animal and display this repertoire on the surface of bacteriophage fd.

Cytoplasmic RNA was isolated according to example 14 from the pooled spleens of five male Balb/c mice boosted 8 weeks after primary immunisation with 2-phenyl-5-oxazolone (ph OX) coupled to chicken serum albumin. cDNA preparation and PCR assembly of the mouse VH and VL kappa repertoires for phage display was as described in example 14. The molecules thus obtained were ligated into fdCAT2.

Vector fdCAT2 was extensively digested with Not1 and ApaL1., purified by electroelution (Sambrook et al.a989 supra) and 1 µg ligated to 0.5 µg (5 µg for the hierarchial libraries: see example 22) of the assembled scFv genes in 1 ml with 8000 units T4 DNA ligase (New

England Biolabs). The ligation was carried out overnight at 16°C. Purified ligation mix was electroporated in six aliquots into MC1061 cells (W. J. Dower, J. F. Miller & C. W. Ragsdale Nucleic Acids Res. 16 6127-6145 1988) and plated on NZY medium (Sambrook et al. 1989 supra) with 15µg/ml tetracycline, in 243x243 mm dishes (Nunc): 90-95% of clones contained scFv genes by PCR screening.

Recombinant colonies were screened by PCR (conditions as in example 7 using primers VH1BACK and MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (see example 14) followed by digestion with the frequent cutting enzyme BstN1 (New England Biolabs, used according to the manufacturers instructions). The library of $2x10^5$ clones appeared diverse as judged by the variety of digestion patterns seen in Figure 23, and sequencing revealed the presence of most VH groups (R. Dildrop, Immunol. Today 5 85-86. 1984) and VK subgroups (Kabat. E.A. et al. 1987 supra) (data not shown). None of the 568 clones tested bound to phOx as detected by ELISA as in example 9.

Thus the ability to select antibody provided by the use of phage antibodies (as in example 21) is essential to readily isolate antibodies with antigen binding activity from randomly combined VH and VL domains. Very extensive screening would be required to isolate antigen-binding fragments if the random combinatorial approach of Huse et al. 1989 (supra) were used.

Example 21

Selection of Antibodies Specific for 2-phenyl-5-oxazolone from a Repertoire Derived from an Immunised Mouse

The library prepared in example 20 was used to demonstrate that ability of the phage system to select antibodies on the basis of their antibody specificity.

None of the 568 clones tested from the unselected library bound to phOx as detected by ELISA.

Screening for binding of the phage to hapten was carried out by ELISA: 96-well plates were coated with 10 µg/ml phOx-BSA or 10 µg/ml BSA in phosphate-buffered saline (PBS) overnight at room temperature. Colonies of phage-transduced bacteria were inoculated into 200 µl 2 x TY with 12.5 µg/ml tetracycline in 96-well plates ('cell wells', Nuclon) and grown with shaking (300 rpm) for 24 hours at 37°C. At this stage cultures were saturated and phage titres were reproducible (10^{10} TU/ml). 50 µl phage supernatant, mixed with 50 µl PBS containing 4% skimmed milk powder, was then added to the coated plates. Further details as in example 9.

The library of phages was passed down a phOx affinity column (Table 4A), and eluted with hapten. Colonies from the library prepared in example 22 were scraped into 50ml 2 x TY medium[37] and shaken at 37°C for 30 min. Liberated phage were precipitated twice with polyethylene glycol and resuspended to 10^{12} TU

(transducing units)/ml in water (titred as in example 8). For affinity selection, a 1 ml column of phOx-BSA-Sepharose (O. Makela, M. Kaartinen, J.L.T. Pelonen and K. Karjalainen J. Exp. Med. 148 1644-1660, 1978) was washed with 300 ml phosphate-buffered saline (PBS), and 20 ml PBS containing 2% skimmed milk powder (MPBS). 10^{12} TU phage were loaded in 10 ml MPBS, washed with 10 ml MPBS and finally 200 ml PBS. The bound phage were eluted with 5 ml 1 mM 4-ϵ-amino-caproic acid methylene 2-phenyl-oxazol-5-one (phOx-CAP; O. Makela et al. 1978, supra). About 10^6 TU eluted phage were amplified by infecting 1 ml log phase E.coli TG1 and plating as above. For a further round of selection, colonies were scraped into 10 ml 2 x TY medium and then processed as above. Of the eluted clones, 13% were found to bind to phOx after the first round selection, and ranged from poor to strong binding in ELISA.

To sequence clones, template DNA was prepared from the supernatants of 10 ml cultures grown for 24 hours, and sequenced using the dideoxy method and a Sequenase kit (USB), with primer LINKFOR (see example 14) for the VH genes and primer fdSEQ1 (5'-GAA TTT TCT GTA TGA GG) for the Vk genes. Twenty-three of these hapten-binding clones were sequenced and eight different VH genes (A to H) were found in a variety of pairings with seven different Vk genes (a to g) (Fig. 24). Most of the domains, such as VH-B and Vk-d were 'promiscuous', able to bind hapten with any of several partners.

The sequences of the V-genes were related to those seen in the secondary response to phOx, but with differences (Fig. 24). Thus phOx hybridomas from the secondary response employ somatically mutated derivatives of three types of Vk genes - Vkoxl. 'Vkox-like' and Vk45.1 genes (C. Berek, G. M. Griffiths & C. Milstein Nature 316 412-418 (1985). These can pair with VH genes from several groups, from Vkoxl more commonly pairs with the VHoxl gene (VH group 2. R.Dildrop uupra). Vkoxl genes are always, and Vkox-like genes often, found in association with heavy chains (including VHoxl) and contain a short five residue CDR3, with the sequence motif Asp-X-Gly-X-X in which the central glycine is needed to create a cavity for phOx. In the random combinatorial library however, nearly all of the VH genes belonged to group 1, and most of the Vk genes were ox-like and associated with VH domains with a five residue CDR3, motif Asp/Asn-X-Gly-X-X (Fig. 24). Vkoxl and VHoxl were found only once (Vk-f and VH-E), and not in combination with each other. Indeed Vk-f lacks the Trp91 involved in phOx binding and was paired with a VH (VH-C) with a six residue CDR3.

A matrix combination of VH and VK genes was identified in phOx-binding clones selected from this

random combinational library. The number of clones found with each combination are shown in Fig. 25. The binding to phOx-BSA, as judged by the ELISA signal, appeared to vary (marked by shading in Fig. 25). No binding was seen to BSA alone.

A second round of selection of the original, random combinational library from immune mice resulted in 93% of eluted clones binding phOx (Table 4). Most of these clones were Vk-d combinations, and bound strongly to phOx in ELISA (data not shown). Few weak binders were seen. This suggested that affinity chromatography had not only enriched for binders, but also for the best.

Florescence quench titrations determined the Kd of VH-B/Vk-d for phOx-GABA as 10^{-8} M (example 23), indicating that antibodies with affinities representative of the secondary response can be selected from secondary response, only two (out of eleven characterised) secrete antibodies of a higher affinity than VH-B/Vk-d (C. Berek et al. 1985 supra). The Kd of VH-B/Vk-b for phOx-GABA was determined as 10^{-5} M (example 23). Thus phage bearing scFv fragments with weak affinities can be selected with antigen, probably due to the avidity of the nultiple antibody heads on the phage.

This example shows that antigen specificities can be isolated from libraries derived from immunised mice. It will often be desired to express these antibodies in a soluble form for further study and for use in therapeutic and diagnostic applications. Example 23 demonstrates determination of the affinity of soluble scFv fragments selected using phage antibodies. Example 27 demonstrates that soluble fragments have similar properties to those displayed on phage. For many purposes it will be desired to construct and express an antibody molecule which contains the Fc portions of the heavy chain, and perhaps vary the immunoglobulin isotype. To accomplish this, it is necessary to subclone the antigen binding sites identified using the phage selection system into a vector for expression in mammalian cells, using methodology similar to that described by Orlandi, R. et al. (1989, supra). For instance, the VH and VL genes could be amplified separately by PCR with primers containing appropriate restriction sites and inserted into vectors such as pSV-gpt HuIgG1 (L. Riechmann et al Nature 332 323-327), 1988) which allows expression of the VH domain as part of a heavy chain IgG1 isotype and pSV-hyg HuCK which allows expression of the VL domain attached to the K light chain constant region. Furthermore, fusions of VH and VL domains can be made with genes encoding non-immunoglobulin proteins, for example, enzymes.

Example 22

Generation of Further Antibody Specificities by the Assembly of Hierarchical Libraries

Further antibody specificities were derived from the library prepared and screened in examples 20 and 21 using a hierarchical approach.

The promiscuity of the VH-B and Vk-d domains prompted the applicants to force further pairings, by assembling these genes with the entire repertoires if either Vk or VH genes from the same immunised mice. The resulting 'hierarchical' libraries, (VH-B x Vk-rep and VH-rep x Vk-d), each with $4x10^7$ members, were subjected to a round of selection and hapten-binding clones isolated (Table 4). As shown by ELISA, most were strong binders. By sequencing twenty-four clones from each library, the applicants identified fourteen new partners for VH-B and thirteen for Vk-d (Fig. 24). Apart from VH-B and Vk-c, none of the previous partners (or indeed other clones) from the random combinatorial library was isolated again. Again the Vk genes were mainly ox-like and the VH genes mainly group 1 (as defined in Dildrop, R. 1984 supra), but the only examples of Vkoxl (Vk-h, -p, -q and -r) have Trp91, and the VH-CDR3 motif Asp-X-Gly-X-X now predominates. Thus some features of the phOx hybridomas seemed to emerge more strongly in the hierarchial library. The new partners differed from each other mainly by small alterations in the CDRs, indicating that much of the subtle diversity had remained untapped by the random combinatorial approach. More generally it has been shown that a spectrum of related antibodies can be made by keeping one of the partners fixed and varying the other, and this could prove invaluable for fine tuning of antibody affinity and specificity.

Therefore, again, phage antibodies allow a greater range of antibody molecules to be analysed for desired properties.

This example, and example 21, demonstrate the isolation of individual antibody specificities through display on the surface of phage. However, for some purposes it may be more desirable to have a mixture of antibodies, equivalent to a polyclonal antiserum (for instance, for immunoprecipitation). To prepare a mixture of antibodies, one could mix clones and express soluble antibodies or antibody fragments or alternatively select clones from a library to give a highly enriched pool of genes encoding antibodies or antibody fragments directed against a ligand of interest and express antibodies from these clones.

Example 23

Selection of Antibodies Displayed on Bacteriophage with Different Affinities for 2-phenyl-5-oxazolone using Affinity Chromatography

The ELISA data shown in example 21 suggested that affinity chromatography had not only enriched for binders, but also for the best. To confirm this, the

binding affinities of a strong binding and a weak binding phage were determined and then demonatrated that they could be separated from each other using affinity chromatography.

Clones VH-B/Vk-b and VH-B/Vk-d were reamplified with MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (see example 14) and VH1BACK-Sfil (5'-TCG CGG CCC AGC CGG CCA TGG CC(G/C) AGG T(C/G)(A/C) A(A/G)C TGC AG(C/G) AGT C(A/T)G G), a primer that introduces an SfiI site (underlined) at the 5' end of the VH gene. VH-B/Vk-d was cloned into a phagemid e.g. pJM1 (a gift from A. Griffiths and J. Marks) as an SfiI-NotI cassette, downstream of the pelB leader for periplasmic secretion (M. Better at al. supra), with a C-terminal peptide tag for detection (see example 24 and figure), and under th control of a P_L promoter (H. Shimatake & M. Rosenberg Nature 292 128-132 1981). The phagemid should have the following features: a) unique SfiI and NotI restriction sites downstream of a pelB leader; b) a sequence encoding a C-terminal peptide tag for detection; and c) a λ P_L promoter controlling expression. 10 litre cultures of E.coli N4830-1 (M. E. Gottesman, S. Adhya & A. Das J.Mol.Biol 140 57-75 1980) harbouring each phagemid were induced as in K. Nagai & H. C. Thogerson (Methods Enzymol 153 461-481 1987) and supernatants precipitated with 50% ammonium sulphate. The resuspended precipitate was dialysed into PBS + 0.2 mM EDTA (PBSE), loaded onto a 1.5ml column of phOx:Sepharose and the column washed sequentially with 100 ml PBS: 100 ml 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0: 10ml 50 mM citrate, pH 5.0: 10 ml 50 mM citrate, pH4.0, and 20 ml 50 mM glycine, pH 3.0. scFv fragments were eluted with 50 mM glycine, pH 2.0, neutralised with Tris base and dialysed against PBSE. VH-B/Vk-b was cloned into a phagemid vector based on pUC119 encoding identical signal and tag sequences to pJM1, and expression induced at 30°C in a 10 litre culture of E.coli TG1 harbouring the phagemid as in D. de Bellis & I. Schwartz (1980 Nucleic Acids Res 18 1311). The low affinity of clone VH-B/Vk-b made its purification on phOx-Sepharose impossible. Therefore after concentration by ultrafiltration (Filtron, Flowgen), the supernatant (100 ml of 600 ml) was loaded onto a 1 ml column of protein A-Sepharose cpoupled (E. Harlow & D. Lane 1988 supra) to the monoclonal antibody 9E10 (Evan, G. I. et al. Mol.Cell Biol.5 3610-3616 1985) that recognises the peptide tag. The column was washed with 200 ml PBS and 50 ml PBS made 0.5 M in NaCl. scFv fragments were eluted with 100 ml 0.2M glycine, pH 3.0, with neutralisation and dialysis as before.

The Kd ($1.0 \pm 0.2 \times 10^{-8}$ M) for clone VH-B/Vk-d was determined by fluorescence quench titration with 4-E-amino-butyric acid methylene 2-phenyl-oxazol-5-one (phOx-

GABA Co. Makela et al, 1978 supra). Excitation was at 280 nm, emission was monitored at 340 nm and the K_d calculated. The K_d of the low affinity clone VH-B/Vk-b was determined as 1.8± 0.3 x 10^{-5} M (not shown). To minimise light adsorption by the higher concentrations of phOx-GABA required, excitation was at 260 nm and emission was monitored at 304 nm. In addition the fluorescence values were divided by those from a parallel titration of the lysozyme binding Fv fragment D1.3. The value was calculated as in H. N. Eisen Meth.Med.Res. 10 115-121 1964. A mixture of clones VH-B/Vk-b and VH-B/Vk-d, $7x10^{10}$ TU phage in the ratio 20 VH-B/Vk-b : 1 VH-B/Vk-d were loaded onto a phOx-BSA-Sepharose column in 10 ml MPBS and eluted as above. Eluted phage were used to reinfect E.coli TG1, and phage produced and harvested as before. Approximately 10^{11} TU phage were loaded onto a second affinity column and the process repeated to give a total of three column passes. Dilutions of eluted phage at each stage were plated in duplicate and probed separately with oligonucleotides specific for Vk-b (5'GAG CGG GTA ACC ACT GTA CT) or Vk-d (5'-GAA TGG TAT AGT ACT ACC CT). After these two rounds, essentially all the eluted phage were VH-B/Vk-d (table 4). Therefore phage antibodies can be selected on the basis of the antigen affinity of the antibody displayed.

Example 24

Construction of Phagemid pHEN1 for the Expression of Antibody Fragments Expressed on the Surface of Bacteriophage following Superinfection

The phagemid pHEN1 (figure 26) is a derivative of pUC119 (Vieira, J. & Messing, J. Methods Enzymol 153 pp 3-11, 1987). The coding region of g3p from fdCAT2, including signal peptide and cloning sites, was amplified by PCR, using primers G3FUFO and G3FUBA (given below) (which contain EcoRI and HindIII sites respectively), and cloned as a HindIII-EcoRI fragment into pUC119. The HindIII-NotI fragment encoding the g3p signal sequence was the replaced by a pelB signal peptide (Better, M. et al. Science 240 1041-1043, 1988) with an internal SfiI site, allowing antibody genes to be cloned as fiI-NotI fragments. A peptide tag, c-myc, (Munro, S. & Pelham, H. Cell 46 291-300, 1986) was introduced directly after the NotI site by cloning an oligonucleotide cassette, and followed by an amber codon introduced by site-directed mutagenesis using an in vitro mutagenesis kit (Amersham International) (figure 26b).

G3FUFO,5'-CAG TGA ATT CTT ATT AAG ACT CCT TAT TAC GCA GTA TGT TAG C;

G3FUBA,5'-TGC GAA GCT TTG GAG CCT TTT TTT TTG GAG ATT TTC AAC G;

Example 25
Display of Single Chain Fv and Fab Fragments Derived from the Anti-Oxazolone Antibody NQ10.12.5 on Bacteriophage fd using pHEN1 and fdCAT2

A range of constructs (see figure 27) were made from a clone (essentially construct II in pUC19) designed for expression in bacteria of a soluble Fab fragment (Better et al. 1988 see above) from the mouse anti-phOx (2-phenyl-5-oxazolone) antibody NQ10.12.5 (Griffiths, G. M. et al. Nature 312, 271-275, 1984). In construct II, the V-regions are derived from NQ10.12.5 and attached to human Ck and CH1 (γ1 isotype) constant domains. The C-terminal cysteine residues, which normally form a covalent link between light and heavy antibody chains, have been deleted from both the constant domains. To clone heavy and light chain genes together as Fab fragments (construct II) or as separate chains (constructs III and IV) for phage display, DNA was amplified from construct II by PCR to introduce a NotI restriction site at the 3' end, and at the 5' end either an ApaLI site (for cloning into fd-CAT2) or SfiI sie (for cloning into pHEN1). The primers FABNOTFOK with VH1BACKAPA (or VH1BACKSFI15) were used for PCR amplification of genes encoding Fab fragments (construct II), the primers FABNOTFOH with VH1BACKAPA (or VH1BACKSFI15) for heavy chains (construct III), and the primers FABNOTFOK and MVKBAAPA (or MVKBASFI) for light chains (construct IV).

The single-chain Fv version of NQ10.12.5 (construct I) has the heavy (VH) and light chain (Vk) variable domains joined by a flexible linker $(Gly_4Ser)_3$ (Huston, J. S. et al. Proc. Natl. Acad. Sci. USA 85 5879-5883, 1988) and was constructed from construct II by 'splicing by overlap extension' as in example 14. The assembled genes were reamplified with primers VK3F2NOT and VH1BACKAPA (or VH1BACKSFI15) to append restriction sites for cloning into fd-CAT2 (ApaLI-NotI) or pHEN1 (SfiI-NotI).

VH1BACKAPA,5'-CAT GAC CAC AGT GCA CAG GT(C/G) (A/C)A(A/G) CTG CAG (C/G)AG TC(A/T) GG;
VH1BACKSFI15,5'-CAT GCC ATG ACT CGC GGC CCA GCC GGC CAT GGC C(C/G)A GGT (C/G)(A/C)A (A/G)CT GCA G(C/G)A GTC (A/T)GG;
FABNOTFOH,5'-CCA CGA TTC TGC GGC CGC TGA AGA TTT GGG CTC AAC TTT CTT GTC GAC;
FABNOTFOK,5'-CCA CGA TTC TGC GGC CGC TGA CTC TCC GCG GTT GAA GCT CTT TGT GAC;
MVKBAAPA,5'-CAC AGT GCA CTC GAC ATT GAG CTC ACC CAG TCT CCA;
MVKBASFI,5'-CAT GAC CAC GCG GCC CAG CCG GCC ATG GCC GAC

ATT GAG CTC ACC CAG TCT CCA;
VK3F2NOT,5'-TTC TGC GGC CGC CCG TTT CAG CTC GAG CTT GGT CCC.
Restriction sites are underlined.

Rescue of Phage and Phagemid particles

Constructs I-IV (figure 27) were introduced into both fd-CAT2 and pHEN1. Phage fd-CAT2 (and fd-CAT2-I,II,III or IV) was taken from the supernatant of infected E.coli TG1 after shaking at 37°C overnight in 2xTY medium with 12.5µg/ml tetracycline, and used directly in ELISA. Phagemid pHEN1 (and pHEN1-I and II) in E.coli TG1 (supE) were grown overnight in 2 ml 2xTY medium, 100 µg/ml ampicillin, and 1% glucose (without glucose, expression of g3p prevents later superinfection by helper phage). 10µl of the overnight culture was used to innoculate 2 ml of 2xTY medium, 100µg/ml ampicillin, 1% glucose, and shaken at 37°C for 1 hour. The cells were washed and resuspended in 2xTY, 100 µg/ml ampicillin, and aphagemid particles rescued by adding 2 µl (10^8pfu) VCSM13 helper phage (Stratagene). After growth for one hour, 4µl kanamycin (25 mg/ml) was added, and the culture grown overnight. The phagemid particles were concentrated 10-fold for ELISA by precipitation with polyethylene glycol.

ELISA

Detection of phage binding to 2-phenyl-5-oxazolone (phOx) was performed as in example 9. 96-well plates were coated with 10 µg/ml phOx-BSA or 10 µg/ml BSA in PBS overnight at room temperature, and blocked with PBSS containing 2% skimmed milk powder. Phage (mid) supernatant (50 µl) mixed with 50 µl PBS containing 4% skimmed milk powder was added to the wells and assayed. To detect binding of soluble scFv or Fab fragments secreted from pHEN1, the c-myc peptide tag described by Munro and Pelham 1986 supra, was detected using the anti-myc monoclonal 9E10 (Evan, G. I. et al. Mol Cell Biol 5 3610-3616, 1985) followed by detection with peroxidase-conjugated goat anti-mouse immonoglobulin. Other details are as in example 9.

The constructs in fdCAT2 and pHEN1 display antibody fragments of the surface of filamentous phage. The phage vector, fd-CAT2 (figure 8) is based on the vector fd-tet (Zacher, A. N. et al. Gene 9 127-140, 1980) and has restriction sites (ApaLI and NotI) for cloning antibody genes (or other protein) genes for expression as fusions to the N-terminus of the phage coat protein g3p. Transcription of the antibody-g3p fusions in fd-CAT2 is driven from the gene III promoter and the fusion protein targetted to the periplasm by means of the g3p leader. Fab abd scFv fragments of NQ10.12.5 cloned into fd-CAT2 for display were shown to bind to phOx-BSA (but not BSA) by ELISA (table 5). Phage were considered to be binding if A_{405} of the sample was at least 10-fold greater that

the background in ELISA.

The phagemid vector, pHEN1 (fig. 26), is based upon pUC119 and contains restriction sites (SfiI and NotI) for cloning the fusion proteins. Here the transcription of antibody-g3p fusions is driven from the inducible lacZ promoter and the fusion protein targetted to the periplasm by means of the pelB leader. Phagemid was rescued with VCSM13 helper phage in 2xTY medium containing no glucose or IPTG: under these conditions there is sufficient expression of antibody-g3p. Fab and scFv fragments of NQ10.12.5 cloned into pHEN1 for display were shown to bind to phOx-BSA (but not BSA) by ELISA (Table 5) using the same criterion as above.

An alternative methodology for preparing libraries of Fab fragments expressed on the surface of phage would be to:

1. Prepare a library of phage expressing heavy chain (VHCH) genes from inserts in the phage genome.
2. Prepare a library of light chain genes in a plamid expression vector in E.coli, preferably a phagemid, and isolate the soluble protein light chins expresed from this library.
3. Bind the soluble protein light chains fromt he library to the heavy chain library displayed on phage.
4. Select phage with the desired properties of affinity and specificity.

These will encode the heavy chain (VHCH) genes.

5. Isolate the light chain genes encoding ight chains which form suitable antigen binding sites in combination with the selected heavy chains, preferably by using superinfectin of bacteria, containing phagemid expressing the light chain, with phage expressing the selected heavy chain (as described in example 20) and then assaying for antigen binding.

Example 26

Rescue of Phagemid Encoding a Gene III Protein Fusion with Antibody Heavy or Light Chains by Phage Encoding the Complementary Antibody Chain Displayed on Phage and the Use of this Technique to Make Dual Combinatorial Libraries

With random combinatorial libraries there is a limitation on the potential diversity of displayed Fab fragments due to the transformation efficiency of bacterial cells. Described here is a strategy (dual combinatorial libraries) to overcome this problem, potentially increasing the number of phage surveyed by a factor of 10^7.

For assembly of heavy and light chains expresses from different vectors, phagemid (pHEN1-III or IV) was grown in E.coli HB2151 (a non-supressor strain) to allow production of soluble chains, and rescued as above (example 27) except that helper phage were used

expressing partner chains as fusions to g3p (10^9 TU fd-CAT2-IV or III respectively) and 2 µl tetracycline (12.5 mg/ml) in place of kanamycin.

Separate Vectors to Encode Fab Heavy and Light Chains

The heavy and light chains of Fab fragments can be encoded together in the same vector (example 25) or in different vectors. To demonstrate this the heavy chain (construct III) was cloned into pHEN1 (to provide soluble fragments) and the light chain (construct IV) into fd-CAT2 (to make the fusion with g3p). The phagemid pHEN1-III, grown in E.coli HB2151 (non-supressor) was rescued with fd-CAT2-IV phage, and phage(mid) shown to bind to phOx:BSA, but not to BSA (Table 5). This demonstrates that soluble light chain is correctly associating with the heavy chain anchored to the g3p, since neither heavy chain nor light chain alone bind antigen (Table 5).

Similar results were obtained in the reverse experiment (with phagemid pHEN-1-IV and fd-CAT2-III phage) in which the heavy chain was produced as a soluble molecule and the light chain anchored to g3p (Table 5). Hence a Fab fragment is assembled on the surface of phage by fusion of either heavy or light chain to g3p, provided the other chain is secreted using the same or another vector (figure 28).

The resulting phage population is a mixture of phage abd rescued phagemid. The ratio of the two types of particle was assessed by infecting log phase E.coli TG1 and plating on TYE plates with either 15 µg/ml tetracycline (to select for fd-CAT2) or 100 µg/ml ampicillin (to select for pHEN1). The titre of fd-CAT2 phage was 5×10^{11} TU/ml and the titre of pHEN1 2×10^{10} TU/ml, indicating a packaging ratio of 25 phage per phagemid.

Demonstrated here is an alternative strategy involving display of the heterodimeric antibody Fab fragments on the surface of phage. One of the chains is fused to g3p and the other is secreted in soluble form into the periplasmic space of the E.coli where it associates non-covalently with the g3p fusion, and binds specifically to antigen. Either the light or heavy chain can be fused to the g3p: they are displayed on the phage as Fab fragments and bind antigen (Figure 28). Described are both phage and phagemid vectors for surface display. Phagemids are probably superior to phage vectors for creation of large phage display libraries. Particularly in view of their higher transfection efficiencies (Two to three orders of magnitude higher), allowing larger libraries to be constructed. The phagemid vector, pHEN1 also allows the expression of soluble Fab fragments in non-suppressor E.coli.

Also demonstrated here is that heavy and light

chains encoded on the same vector (construct II), or on different vectors (constructs III and IV) can be displayed as Fab fragments. This offers two distinct ways of making random combinatorial libraries for display. Libraries of heavy and light chain genes, amplified by PCR, could be randomly linked by a 'PCR assembly' process (example 14) based on 'splicing by overlap extension', cloned into phage(mid) display vectors and expressed from the same promoter as part of the same transcript (construct II) as above, or indeed from different promoters as separate transcripts. Here the phage(mid) vector encodes and displays both chains. For a combinatorial library of 10^7 heavy chains and 10^7 light chains, the potential diversity of displayed Fab fragments (10^{14}) is limited by the transfection efficiency of bacterial cells by the vector (about 10^9 clones per µg cut and ligated plasmid at best) (W.J. Dower et al Nucl. Acids. Res. 16 6127-6145, 1988). Libraries thus prepared are analogous to the random combinatorial library method described by Huse, W.D. et al Science 246 1275-1281 (1989), but have the important additional feature that display on the surface of phage gives a powerful method of selecting antibody specificities from the large number of clones generated.

Alternatively, libraries of heavy and light chains could be cloned into different vectors for expression in the same cell, with a phage vector encoding the g3p fusion and a phagemid encoding the soluble chain. The phage acts as a helper, and the infected bacteria produced both packaged phage and phagemid. Each phage or phagemid displays both chains but encodes only one chain and thus only the genetic information for half of the antigen-binding site. However, the genes for both antibody chains can be recovered separately by plating on the selective medium, suggesting a means by which mutually complementary pairs of antigen binding heavy and light chain combinations could be selected from random combinatorial libraries. For example, a light chain repertoire on fd phage could be used to infect cells harbouring a library of soluble heavy chains on the phagemid. The affinity purified phagemid library could then be used to infect E.coli, rescued with the affinity purified phage library, and the new combinatorial library subjected to a further round of selection. Thus, antibody heavy and light chain genes are reshuffled after each round of purification. Finally, after several rounds, infected bacteria could be plated and screened individually for antigen-binding phage. Such 'dual' combinatorial libraries are potentially more diverse than those encoded on a single vector. By combining separate libraries of 10^7 light chain phage(mid)s, the diversity of displayed Fab fragments (potentially 10^{14}) is limited

only by the number of bacteria (10^{12} per litre). More simply, the use of two vectors should also facilitate the construction of 'hierarchical' libraries, in which a fixed heavy or light chain is paired with a library or partners (example 22), offering a means of 'fine-tuning' antibody affinity and specificity.

Example 27

Induction of Soluble scFv and Fab Fragments using Phagemid pHEN1

Further study of antibodies which have been expressed on the surface of phage would be greatly facilitated if it is simple to switch to expression in solution.

E.coli HB2151 was infected with pHEN phagemid (pHEN1-I or II), and plated on YTE, 100µg/ml ampicillin plates. Colonies were shaken at 37°C in 2xTY medium, 100 µg/ml ampicillin, 1% glucose to OD_{550}=0.5 to 1.0. Cells were pelleted, washed once in 2xTY medium, resuspended in medium with 100 µg/ml ampicillin, 1 mM isopropyl β-D-thiogalactoside (IPTG), and grown for a further 16 hours. Cells were pelleted and the supernatant, containing the secreted chains, used directly in ELISA.

The phagemid pHEN1 has the advantage over phage fd-CAT2, in that antibody can be produced either for phage display (by growth in supE strains of E.coli) or as a tagged soluble fragment (by growth in non-suppressor strains), as a peptide tag (example 24) and amber codon were introduced between the antibody and g3p. Secretion of soluble Fab fragments from pHEN1-II or scFv fragments from pHEN1-I was demonstrated after growth in E.coli HB2151 and induction with IPTG using Western blots (Figure 29). For detection of secreted proteins, 10µl supernatant of induced cultures were subjected to SDS-PAGE and proteins transferred by electroblotting to Immobilon-P (Millipore). Soluble heavy and light chain were detected with goat polyclonal anti-human Fab antiserum (Sigma) and peroxidase conjugated rabbit anti-goat immunoglobulin (Sigma), each at a dilution of 1:1000. The tagged VK domain was detected with 9E10 antibody (1:1000) and peroxidase conjugated goat anti-mouse immunoglobulin (Fc specific) (1:1000) (Sigma) or with a peroxidase labelled anti-human CK antiserum (Dako). 3,3'-diaminobenzidine (DAB;Sigma) was used as peroxidase substrate (Harlow E., et al. 1988 Supr). With the scFv, the fragments were detected using the 9E10 anti-myc tag antibody (data not shown). With the Fab, only the light chain was detected by 9E10 (or anti-human CK) antibody, as expected, while the anti-human Fab antiserum detected both heavy and light chains. Binding of the soluble scFv and Fab fragments to phOx-BSA (but not to BSA) was also demonstrated by ELISA (Table 5B). Thus scFv and Fab fragments can be displayed on phage or

secreted as soluble fragments from the same phagemid vector.

Example 28

Increased Sensitivity in ELISA assay of Lysozyme using FDTscFvD1.3 as Primary Antibody Compared to Soluble scFvD1.3

In principle the use of phage antibodies should allow more sensitive immunoassays to be performed than with soluble antibodies. Phage antibodies combine the ability to bind a specific antigen with the potential for amplification through the presence of multiple (ca.2800) copies of the major coat protein (g8p) on each virion. This would allow the attachment of several antibody molecules directed against M13 to each virion followed by the attachment of several molecules of peroxidase-conjugated anti-species antibody (anti-sheep) IgG in the case below). Thus for every phage antibody bound to antigen there is the potential for attaching several peroxidase molecules whereas when a soluble antibody is used as the primary antibody this amplification will not occur.

ELISA plates were coated overnight at room temperature using 200µl of 10 fold dilutions of hen egg lysozyme (1000, 100, 10, 1, 0.1 and 0.01 µg/ml) in 50mM $NaHCO_3$, pH9.6. ELISA was performed as described in example 4 except that (i) incubation with anti-lysozyme antibody was with either FDTscFvD1.3 (pAb;10^{11} phage per well; 1.6mol) or soluble affinity purified scFvD1.3 (18µg per well; 0.7nmol) (ii) incubation with second antibody was with 1/100 dilution of sheep anti-M13 serum for FDTscFvD1.3 samples or with or 1/100 dilution of rabbit anti-scFvD1.3 serum (from S. Ward) for soluble scFvD1.3 samples (iii) peroxidase-conjugated rabbit anti-goat immunoglobulin (Sigma; 1/5000) was used for FDTscFvD1.3 samples and peroxidase-conjugated goat anti-rabbit immunoglobulin (Sigma; 1/5000) was used for soluble scFvD1.3 samples. Absorbance at 405nm was measured after 15h. The results are shown in Figures 30 and 31. In these figures lysozyme concentrations for coating are shown on a log scale of dilutions relative to 1µg/ml. (i.e. log = -3 =1mg/ml ; log = 2 = 0.01 µg/ml)

Higher signals were obtained with FDTscFvD1.3 at all concentrations of lysozyme (Fig.31) but the difference was very marked at the greatest dilutions, where antigen quantities are most limiting (Figs. 30 and 31). This suggests that phage antibodies may be particularly valuable for sandwich type assays where the capture of small amounts of antigen by the primary antibody will generate an amplified signal when phage antibodies directed against a different epitope are used as the second antigen binding antibody.

Example 29

Direct Rescue and Expression of Mouse Monoclonal Antibodies as Single Chain Fv Fragments on the Surface of Bacteriophage fd.

The principle is very similar to that described in example 14. It consists of the PCR assembly of single chain antibodies from cDNA prepared from mouse monoclonals. As an example, the rescue and expression of two such antibodies from monoclonals expressing antibodies against the steroid hormone oestriol is described.

A. RNA Preparation

RNA can be prepared using many procedures well known to those skilled in the art. In this example, the use of Triton X-100 lysis, phenol/SDS RNase inactivation gave excellent results.

1. The mouse monoclonal cells that were used here had been harvested by centrifugation and resuspended in serum free medium. They were then centrifuged and resuspended in saline and after a final centrifugation step, resuspended in sterile water at 1 x 10^7 cells per ml. (Normally cells would be washed in PBS buffer and finally resuspended in PBS buffer, but these particular cells were supplied to us as described frozen in water.).

2. To 750µl of cells was added 250ul of ice cold 4X lysis buffer (40mM Tris HCl pH 7.4/4mM $MgCl_2$/600mM NaCl/40mM VRC (Veronyl ribosyl complex)/2% Triton X-100). The suspension was mixed well and left on ice for 5 minutes.

3. Centrifugation was carried out at 4°C in a microfuge at 13000 rpm for 5 min.

The supernatant is then phenol extracted three times, phenol chloroform extracted three times and finally, ethanol precipitated as described in the materials and methods. The precipitate was resuspended in 50ul water.

4. The optical density of the RNA at 260nm with a 2.5ul sample in 1ml water was measured. The RNA was checked by electrophoresis of a 2ug sample on a 1% agarose gel. RNA in the range of 32ug to 42ug was obtained by this method.

B. cDNA Preparation

The method used is the same as that described in example 14. Two cDNA preparations were made. These were from RNA extracted from the monoclonals known as cell lines 013 and 014 which both express antibodies against eh steroid hormone, oestriol.

C. Primary PCRs

The method used is essentially the same as that described in example 14. The VH region was amplified with the primers VH1BACK and VH1FOR-2. For the Vkappa region, four separate reactions were carried out using the primer VK2BACK and wither MJK1FONX, MJK2FONX, MJK4FONX or MJK5FONX. Samples (5ul) were checked on a 1.5% agarose gel. From this it was observed that for

cDNA prepared from the two oestriol monoclonals the primers VK2BACK and MJK1FONX gave the best amplification of the Vkappa region. The VH bands and the Vkappa bands amplified with VK2BACK/MJK1FONX were purified on 2% low melting point agarose gels for each monoclonals. The DNA bands were excised from the gel and purified using a dedicated Geneclean kit as described in example 14.

D. Preparation of linker

The method used is essentially the same as that described in example 14. In this case, the amplified linker DNA was purified on a 2% agarose gel and recovered from the gel with a dedicated "Mermaid" kit (BIO 101, Geneclean, La Jolla, San Diego, California, USA) using the manufacturers instructions.

E. Assembly PCRs

The method used is essentially the same as that described in example 14. In this case, the assembled PCR product was purified on a 2% agarose gel and recovered from the gel with a dedicated "Mermaid" kit.

F. Adding restriction sites and work-up

The assembled product was "tagged" with Apa LI and Not I restriction sites. The DNA was then digested with Apa LI and Not I to give the appropriate sticky ends for cloning and then purified on a 2% low melting point agarose gel and extracted using a Geneclean kit. The method used is the same as that described in example 14.

G. Cloning into Vector fd-CAT2

A total of 15ug of CsCl purified fd-CAT2 DNA was digested with 100 units of the restriction enzyme Not I (New England Biolabs) in a total volume of 200ul 1X NEB Not I buffer with 1X NEB acetylated BSA for a total of 3 hours at 37°C. The vector DNA was the treated twice with 15ul Strataclean (a commercially available resin for the removal of protein), following the manufacturers instructions (Stratagene, 11099 North Torrey Pines Road, La Jolla, California, USA). The DNA was then ethanol precipitated and redissolved in TE buffer (Sambrook et al., 1989 supra). The DNA was then digested with 100 units of the restriction enzyme Apa LI (New England Biolabs) in a total volume of 200ul 1X NEB Buffer 4 overnight at 37°C. The vector was then purified with a Chroma Spin 1000 column following the manufacturers instructions (Clontech Laboratories Inc, 4030 Fabian way, Palo Alto, California, USA). This step removes the Apa LI/Not I fragment to give cut vector DNA for maximum ligation efficiency.

Ligation reactions were carried out with 2.5-10ng of the DNA insert and 10ng of vector in a total volume of 10ul of 1X NEB ligase buffer with 1ul of NEB ligase (New England Biolabs) at 16°C overnight (approx 16 hours).

H. Transformation and growth

E.coli strain TG1 was made competent and transformed

with the fdCAT2 recombinant DNA as described by Sambrook et al, 1989 Supra. The cells were plated out on LBtet plates (10g tryptone, 5g yeast extract, 10g NaCl, 15g bacto-agar per litre with 15ug/ul of tetracycline added just before pouring the plates) and grown overnight.

Single well isolated colonies were then inoculated into 10 ml of LBtet broth (LB medium with 15ug/ul of tetracycline) in 50 ml tubes. After overnight growth at 35°C/350rpm in a bench top centrifuge. The supernatants were transferred to 15 ml centrifuge tubes and 2ml 20% PEG 8000/2.5M NaCl added to each. After incubating at room temperature for 20-30 minutes, the recombinant phage was pelleted by centrifugation at 9000rpm in a Sorval SM24 rotor for 30 minutes. The PEG supernatant was discarded. Any remaining PEG was removed with a pasteur pepette after a brief (2 minutes) centrifugation step. This last step was repeated to make sure that no PEG remained. The phage pellet was then resuspended in 500ul PBS buffer. This was transferred to a microcentrifuge tube and spun at 13000 rpm to remove any remaining cells. The phage supernatant was transferred to a fresh tube.

I. Assay for antibody expression

Bacteriophage fd recombinants were screened for the expression of antibody against oestriol by ELISA. This method is described in example 6. In this case the following alterations are relevant.

1. Microtitre plates were coated overnight with 40ug/ml oestriol-6 carboxymethyloxime-BSA (Steraloids, 31 Radcliffe Road, Croydon, CRO 5QJ, England).
2. 1st antibody was the putative phage anti oestriol antibody. 50ul of phage in a final volume of 200ul of sterile PBS combining 0.25% gelatin was added to each well.
3. 2nd antibody was sheep anti M13 at 1:1000 dilution.
4. 3rd antibody was peroxidase conjugated rabbit anti goat immunoglobulin.

Recombinants expressing functional antibody were detected by incubation with the chromogenic substrate 2'2' axinobis (3-ethyl benzthiazoline sulphonic acid). The results are shown in figures 32 and 33.

Example 30

Kinetic Properties of Alkaline Phosphatase Displayed on the Surface of Bacteriophage fd

This example demonstrates that kinetic properties of an enzyme expressed on phage are qualitatively similar to those in solution. Bacteriophage fd displaying alkaline phosphatase fusions of gene 3 with either the native arginine (see example 31) or the mutant residue alanine at position 166 (see example 1[illegible]) were prepared by PEG precipitation as described in the materials and methods.

The kinetic parameters of alkaline phosphatase expressed on the surface of fd phage were investigated in

1M Tris/HCl, pH8.0 at 20°C with 1ml 4-nitrophenyl phosphate as substrate. The reactions were initiated by the addition of 100µl of a phage-alkaline phosphatase fusion preparation, 50 fold concentrated with respect to the original culture supernatant. The rate of change of absorbance was monitored at 410nm using a Philips 8730 spectrophotometer and the initial reaction rate calculated using a molar absorbance of 16200 1/mol/cm. For the fdphoAla 166 enzyme but not fdphoArg166 a lag phage was seen following this addition, the reaction rate accelerating until a steady state was obtained after approximately 60 to 90 secs. This steady state rate was used for determination of kinetic parameters. No deviation form Michaelis Menten kinetics was apparent for either phage enzyme. Values of K_m and k_{cat} were derived from plots of s/v against s and are shown in Table 6.

Because of the difficulty in establishing the relationship between the number of phage particles an the number of active enzyme dimers formed on the phage k_{cat} values are expressed not as absolute values, but as relative values between the two enzyme forms. Western blots (carried out as in example 31 using antig3p antiserum) of the phage enzyme preparations used in this experiment showed approximately equal intensities for the full length fusion band with the Arg166 and Ala166 enzymes when detected using antibody directed against gene3. In these preparations the intact fusion represents approximately 30% of the detected material. The two preparations were therefore assumed to be expressing approximately the same concentrations of intact fusions.

Table 6 summarises the kinetic data from this experiment and compares it with data from Chaidaroglou, A. et al (Biochemistry 27, 8338-8343 (1988)) obtained with soluble preparations of the wild type and mutant enzyme forms. The same substrate and assay conditions were used in both experiments. Soluble alkaline phosphatase was also tested in parallel in our experiments (K_m=8.5µM; kcat=3480 mol substrate converted mol $enzyme^{-1}$ min^{-1}).

The effect of mutating arginine at position 166 to alanine is qualitatively similar for the phage enzyme as for the soluble enzyme. K_m is increased about 15 fold and the relative k_{cat} is decreased to 36% of that for wild type. This increased K_m would reflect a reduction in substrate affinity in the phage enzyme on mutation of Arg166, as was proposed for the soluble enzyme (Chaidaroglou et al, 1988 supra), assuming the same kinetic mechanism applies. There are, however, some quantitative differences in the behaviour of K_m of the phage enzyme. The K_m of 73µM observed for fdphoArg166 compares with a K_m of 12.7µM for the free enzyme; the K_m

for fdphoAla166 is 1070μM whereas the free mutant enzyme has a K_m of 1620μM. One can speculate that the higher K_m for fdphoArg 166 and the lower K_m for fdphoAla166, compared to the soluble enzymes result from the 'anchored' alkaline phosphatase fusion molecules interacting to form dimers in a different manner to the enzyme in free solution.

The relative values of k_{cat} for the Arg166 and Ala166 forms are however very similar for both the phage enzymes and the soluble enzymes, a reduction occurring on mutation to 35 to 40% of the value for the native enzyme. The rate limiting step, determining k_{cat}, for soluble phoArg166 is thought to be dissociation of non-covalently bound phosphate from the enzyme (Hull W.E. et al. Biochemistry 15, 1547-1561 1976). Chaidaroglou et al (1988) supra suggest that, for the soluble enzyme, mutation of Arg166 to alanine alters additional steps, one of which may be hydrolysis of the phosphoenzyme intermediate. The similarity in the reduction in k_{cat} on mutation of Arg166 to alanine for the phage enzymes suggests that the same steps may be altered in a quantitatively similar manner in the mutant phage enzyme as in the mutant soluble enzyme.

Thus, enzymes displayed on phage show qualitatively similar characteristics to soluble enzymes.

Example 31

Demonstration using Ultrafiltration that Cloned Alkaline Phosphatase Behaves as Part of the Virus Particle

The construct fdphoAla166 (derived in example 11) was converted back to the wild type residue (arginine) at position 166 by in vitro mutagenesis (Amersham International) using the printer
APARG166:5' TAGCATTTGCGCGAGGTCACA 3'.
This construct with the wild type insert was called fdphoArg166.

E.coli TG1 or KS272 cells (cells with a deletion in the endogenous phoA gene, Strauch and Beckwith, 1988 Supra) containing either fd-phoAla166, fdphoArg166 or fd-CAT2 were grown for 16 hours at 37°C in 2xTY with 15μg/ml tetracycline. Concentrated phage were prepared as follows. Phage-enzyme cultures are clarified by centrifugation (15 min at 10,000 rpm, 8 x 50 ml rotor, sorval RC-5B centrifuge). Phage are precipitated by adding 1/5 volume 20% polyethylene glycol, 2.5 M Nacl, leaving for 1 hr at 4°C, and centrifuging (as above). Phage pellets are resuspended in 10 mM Tris-HCl, pH 8.0 to 1/100th of the original volume, and residual bacteria and aggregated phage removed by centrifugation for 10 to 15 minutes in a bench microcentrifuge at 13000 rpm at 4°C.

SDS/Polyacrylamide gel electrophoresis and western blotting were basically as described previously (example

2). Denatured samples consisting of 16µl of a 50 fold concentrate of phage were separated using a 10% SDS/polyacrylamide gel and detected with polyclonal antiserum raised against either E.coli alkaline phosphatase (Northumbria Biologicals, South Nelson Industrial Estate, Cramlington, Northumberland, NE23 9HL) or against the minor coat protein encoded by gene 3 (from Prof. I. Rasched, Universitat Konstanz, see Stengele et al, 1990) at 1 in 1000 dilution. This was followed by incubation with peroxidase-conjugated goat-anti-rabbit immunoglobulin (Sigma 1 in 5000) and detection with the ECL Western blotting system (Amersham International).

The presence of fusion proteins was confirmed by western blotting of proteins from phage particles derived from fd-phoAla166 (phage-enzyme) or fd-CAT2 (vector phage). Detection with antiserum raised against the gene 3 protein reveals a product of apparent relative molecular mass (Mr) of 63,000 in vector phage (figure 34e). Although this is different from the predicted molecular weight based on the amino acid sequence (42,000), the natural product of gene 3 has previously been reported to exhibit reduced mobility during electrophoresis (Stengele et al, 1990).

In the fd-phoAla166 sample the largest band has an apparent Mr of 115,000, (fig. 34). Taking into account the aberrant mobility of the gene 3 portion of the fusion, this is approximately the size expected from fusing with an alkaline phosphatase domain of 47 kD. This analysis also reveals that a proportion of the Gene3 reactive material in this phage-enzyme preparation is present at the size of the native gene3 product, suggesting that degradation is occurring. In the preparation shown in figure 34, approximately 5-10% of the gene 3 fusions are intact. In more recent preparations and in all the preparations used in this example and example 32, approximately 30-60% of fusions are full length.

The protein of Mr 115,000 is the major protein observed in Western blots of phage-enzyme derived from TG1 cells when probed with antiserum raised against E.coli alkaline phosphatase (anti-BAP), confirming the assignment of this band to intact fusion. Further, when phage enzyme is prepared using KS272 cells, which have a deletion in the endogenous phoA gene (Strauch & Beckwith, 1988, supra.) it is also the major band. There are additional bands at Mr 95000 and 60000 reactive with anti-BAP antiserum which may indicate degradation of the fusion product.

The anti-BAP antiserum also reacts wit material running with the dye front and with a molecule of Mr 45,000 but evidence suggests that this material is not alkaline phosphatase. This pattern is detected in PEG

precipitated vector phage samples (figure 34c) and is not therefore contributed by protein expressed from the cloned phoA gene. These bands are detected in culture supernatants of cells carrying fd-CAT2 but is not detected in the supernatant of uninfected cells (not shown) and so either represents cross-reactivity with phage encoded material or with a PEG precipitable cellular component leaked from infected cells (Boeke et al, Mol. Gen. Genet. 186, 185-192 1982). Although the fragment of Mr, 45,000 is close to the size of free alkaline phosphatase (47,000), it is present in phage preparations from KS272 cells which have a deletion in the phoA locus. Furthermore its mobility is different from purified alkaline phosphatase and they can be distinguished by electrophoresis (figure 34d).

Ultrafiltration was used to confirm that the fusion protein behaved as though it were part of a larger structure, as would be expected for an enzyme bound to a phage particle. Phage samples (100µl of a 50 fold concentrate) were passed through ultrafiltration filters with a nominal molecular weight limit of 300000 daltons (Ultrafree-MC filters, Millipore) by centrifugation for 5 to 15 minutes at 13,000 r.p.m. in an MSE microcentaur microfuge. Retained material was recovered by resuspending in 100µl of 10mM Tris, pH 8.0.

Phage-enzyme or free alkaline phosphatase (83ng) mixed with vector phage were passed through filters with a nominal molecular weight limit of 300,000 daltons (Ultrafree-MC filters, Millipore). Figure 35 A again shows that the band of Mr, 115,000 is the major product reactive with anti-BAP antiserum. This and the other minor products reactive with anti-BAP are present in material retained by the ultrafiltration membrane. Analysis of retained and flow through fractions of phage preparations derived from KS272 demonstrates that different molecular species are being separated by the ultrafiltration membranes. Figure 35b shows the protein of Mr 115,000 is retained by the filter whereas the putative degradation products of Mr 95,000 and 60,000 found in phage preparations derived from KS272 cells, are not retained.

In mixture of alkaline phosphatase and vector phage Figure 35c-f, free alkaline phosphatase (dimer size of 94,000 daltons) is detected in the flow through as a monomer band with Mr 47,000 on denaturing polyacrulamide gels (figure 35B), while the cross reactive molecule found in vector phage preparations (Mr 45,000) is in retained on the filter (figure 35B). This suggests that the cross reactive molecule is part of the phage particle and underlines the fact that the ultrafiltration membranes are effecting a separation. Thus the expected fusion band in this phage-enzyme is present in material

retained on ultrafiltration membranes demonstrating that it is part of a larger structure as would be expected for viral bound enzyme.

Catalytic activity has been demonstrated on phage particles expressing alkaline phosphatase. Table 7 shows that the wild type alkaline phosphatase gene expressed on phage (fd-phoArg166) has a specific activity (moles of substrate converted per mole of viral particles) of 3,700/min. This is close to the turnover value of 4540/min found for purified alkaline phosphatase by Malamy and Horecker, Biochemistry 3, 1893-1897 1964).

Chaidaroglou et al, 1988 supra have shown that substituting alanine for arginine at the active site (residue 166) leads to a reduction in the rate of catalysis. Preparations of phage displaying alkaline phosphatase with this mutation derived from TG1 and KS272 show reduced specific activities of 380 and 1400 mol substrate converted/mol phage/min respectively. Enzyme activity was measured in the retained and flow-through fractions prepared by ultrafiltration, shown in figure 35. The bulk of activity from phage-enzyme was retained on the filters whereas the majority of activity from free enzyme passes through. Therefore, the enzyme activity in these fusions behaved as would be expected for virally associated enzyme (not shown). Little or no catalytic activity is measured in preparations of vector phage from either TG1 or KS272 cells (Table 7), indication that the catalytic activities above are due to phage enzyme and not contamination with bacterial phosphatase. Addition of phage particles to soluble enzyme does not have a significant effect on activity (Table 7).

Therefore, both the catalytic and immunochemical activity of alkaline phosphatase have been demonstrated to be due to enzyme which is part of the phage particle.

Example 32

Affinity chromatography of phage alkaline phosphatase

Affinity chromatography, using the specific binding properties of enzymes has proved to be a very powerful method for their purification. The purification of phage-enzymes by this approach would enable the genetic material encoding the enzyme to be isolated with the enzyme itself. Thus, mutagenesis of cloned enzymes expressed on the surface of filamentous bacteriophage will lead to a whole population of enzyme variants, from which variants with desired binding properties could be isolated.

Soluble alkaline phosphatase (from calf intestine) has been purified by binding to immobilised arsenate (a competitive inhibitor), and eluting with inorganic phosphate, which is a product (and competitive inhibitor) of the enzyme reaction (Brenna,O. et al, Biochem. J. 151 291-296 1975). The applicants have determined that

soluble alkaline phosphatase from E.coli is also retained by this matrix (not shown). In this example it is demonstrated that phage displaying E.coli alkaline phosphatase binds to arsenate-Sepharose and can be specifically eluted.

Arsenate-Sepharose was prepared by coupling 4-(p-aminophenylazo) phenyl arsonic acid to tyraminyl-Sepharose according to the method of Breena et al, (1975; supra). Affinity chromatography of phage enzyme fdphoArg166 (example 31) was carried out in a disposable chromatography column with a 0.5 ml column volume. Columns were prewashed with 100 volumes of column buffer (100mM Tris pH 8.4, 1mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1% Tween 20, Brenna et al, 1975, supra.) 1ml of a 40 fold concentrate of phage-enzyme (in column buffer; prepared as in example 31) was loaded and washed through with 100 volumes of column buffer. Bound phage-enzyme was eluted with 5mls of column buffer containing 20mM $NaHPO_4$. The eluate and wash fractions were quantitated by dot blotting onto nitrocellulose and comparing with known amounts of phage-enzyme. The blots were detected using sheep anti-M13 antiserum (gift from M. Hobart), anti-sheep peroxidase (Sigma) and enhanced chemiluminescent substrate (Amersham). A range of exposures were taken.

Table 8 shows the results of affinity chromatography of phage displaying alkaline phosphatase on arsenate-Sepharose. In separate experiments phage particles expressing either mutant (fdphoAla 166; example 11) and or wild type (fdphoArg 166) forms are retained on arsenate-Sepharose and eluted with inorganic phosphate. Approximately 0.5 to 3% of added phage enzyme particles loaded ('input phage') were specifically eluted with phosphate ('output phage') compared to only 0.05% of vector particles. Arsenate is a competitive inhibitor with K_i of 20μM with respect to 4- nitrophenyl phosphate. Phage particles antibodies have previously been isolated on the basis of interactions with similar affinities (example 23). This association is in within the range of a large number of enzyme-ligand interactions suggesting wide applicability for this approach.

Table 8 also shows that the infectivity of phage particles expressing enzyme is reduced with compared with vector phage particles. This makes titration of infectious particles an inappropriate means of quantitating the number of phage enzyme particles. For this reason the number of phage were measured by dot blotting and phage were detected with anti-M13 antiserum as above.

Whereas, overall recovery of catalytic activity may be an important consideration in enzyme purification, this is not critical with phage-enzymes. Even if only low levels of phage-enzyme bind to and are specifically

eluted from affinity columns, this will generate clones which can subsequently be grown up in bulk as phage-enzymes or can be transferred to expression vectors yielding soluble products.

Example 33

PCR Assembly of DNA encoding Fab Fragments of an Antibody directed against Oxazolone

Example 25 showed that genes encoding Fab fragments could be subcloned into vectors fdCAT2 and pHEN1 and the protein domains displayed on the surface of phage with retention of binding function. This example shows that the VHCH and VKCK domains can be amplified separately and then joined by a linker allowing the expression of the light chain as a geneIII protein fusion and the VHCH fragment as a soluble molecule. A functional Fab fragment is then displayed on phage by association of these domains. The assembly process, described in this example, is required for display of a library of Fab fragments derived from the immune repertoire if both heavy and light chain domains are to be encoded within a single vector.

The VHCH1 and VKCK domains of a construct (example 25; construct II in pUC19) derived from antibody NQ10 12.5 directed against 2-phenyl-5-oxazolone were amplified using PCR. For cloning into the vector fdCAT2 the oligonucleotides VH1BACKAPA (example 25) and HuIgG1-4 CH1FOR (example 40) were used to amplify the VHCH1 domains. For cloning into pHEN1 VH1BACKSFH5 (example 25) replaced VH1BACKAPA for this amplification. For cloning into both vectors the VKCK domains were amplified using VK2BACK (example 25) and CKNOTFOR (example 40). A linker oligonucleotide fragment containing the bacteriophage fd gene 8 terminator and the fd gene 3 promoter was prepared by amplifying the region containing them from the vector fdCAT2 by PCR using the oligonucleotides.

VK-TERM-FOR

5' TGG AGA CTG GGT GAG CTC AAT GTC GGA GTG AGA ATA GAA AGG 3' (overlapping with VK2BACK [example 14])

and

CH1-TERM-BACK

5'AAG CCC AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG CCC AAA TCT AGC TGA TAA ACC GAT ACA ATT AAA GGC 3' (overlapping with HuIgG1-4 CH1-FOR)

Assembly of the Fab fragment from the amplified VHCH1 and VKCK domains and the linker prepared as above was as described in example 14E except that the primers VH1BACKAPA (when cloning into fdCAT2) or VH1BACKSFH5 (when cloning into pHEN1) and CKNOTFOR were used for the final reamplification, thereby introducing restriction sites for cloning into fdCAT2 (ApaLI-NotI) or pHEN1 (SfiI-NotI) the assembled Fab fragment is shown in figure 34. No assembled product was seen in the absence of

linker. An assembled scFv prepared according to example 14 is shown for comparison.

Phage antibodies were prepared as in example 25 and ELISA was performed with oxazolone as antigen according to example 6. Results were as expected for Fab fragments cloned in both fdCAT2 and pHEN1 samples, phage particles bound to oxazolone as detected by a positive ELISA signal.

Example 34

Construction of a Gene III Deficient Helper Phage

To fully realise the potential of the phagemid cloning system, a helper phage lacking gene III is desirable. Rescue of gene III fusions with such a helper phage would result in all the progeny phagemids having a gene III fusion on their capsid, since there would be no competition with the wild type molecule.

Control over the number of fusion molecules contained on each phage will provide particularly useful. For example, a gene III deficient helper phage can be used to rescue low affinity antibodies from a naive repertoire, in which high avidity will be necessary to isolate those phage bearing the correct antibody specificity. The unmutated helper phage can then be used when higher affinity versions are constructed, thereby reducing the avidity component, and permitting selection purely on the basis of affinity. This will prove a surprisingly successful strategy for isolation and affinity maturation of antibodies from naive libraries.

The strategy chosen to construct the helper phage was to partially delete gene III of M13KO7 using exonuclease Bal 31. However, phage lacking gene III protein are non-infective so an E.coli strain expressing gene III was constructed. Wild type M13 gene III was PCR-amplified with primers gIIIFUFO and gIIIFUBA, exactly as described in example 24. The PCR product was digested with Eco RI and Hind III and inserted into Eco RI and Hind III-cut pUC19 (not a phagemid as it lacks the filamentous phage origin of SS DNA replication) under control of the lac promoter. The plasmid was transformed into E.coli TG1, and the resulting strain called TG1/pUC19gIII. This strain provides gIII protein in trans to the helper phage.

There is a single unique Bam HI site in M13KO7, which is approximatlely in the centre of gIII. Double-stranded M13KO7 DNA was prepared by alkaline lysis and caesium chloride centrifugation (Sambrook et al, et supra. 1989); twenty µg of DNA was cut with Bam H1, phenol extracted and ethanol precipitated then resuspended in 50µl of Bal 31 buffer (600mM NaCl, 20mM Tris-HCl pH 8.0, 12 mM $CaCl_2$, 12mM $MgCl_2$ and 1mM EDTA) and digested for 4 minutes with 1 unit of Bal 31 (New England BioLabs). This treatment removed approximatley

1Kb of DNA. EGTA was added to 20mM and the reaction phenol extracted and ethanol precipitated prior to purification of the truncated genome on an agarose gel. The DNA was repaired with klenow enzyme and self-ligated with T4 DNA ligase (New England BioLabs).

Aliquots of the ligation reaction were transformed into competent TG1/pUC19gIII and plated on SOB medium containing ampicillin at 100µg/ml and kanamycin at 50µg/ml. Colonies were screened for the presence of a deletion by PCR with primers gIIIFUBA and KSJ12 (CGGAATACCCAAAAGAACTGG).

KSJ 12 anneals to gene VI which is immediately downstream of gIII in the phage genome, so distinguishing gIII on the helper phage from that resident on the plasmid. Three clones gave tructated PCR products corresponding to deletions of ca. 200, 400 and 800bp. These clones were called M13K07 gIII Δ Nos 1,2 and 3 respectively. No clones were isolated from the earlier Bal 31 time points, suggesting that these are in some way lethal to the host cell. Several clones were isolated from later time points, but none of these gave a PCR product, indicating that the deletion reaction had gone too far.

M13K07 gIII Δ No.s 1,2 and 3 were cultured and the resulting helper phage tested for their ability to rescue an antibody gIII fusion (scFv D1.3) by ELISA, exactly as described in example 18. As shown in figure 37, only one clone, M13K07 gIIIΔ No3 was found to rescue the antibody well; in fact the signal using this helper was greater than that observed with the parent M13 K07. M13K07 gIIIΔ No3 rescued phagemids should have a much higher density of antibody fusions on their surfaces. That this was indeed the case was demonstrated when the phage used in this ELISA were analysed by Western blotting with anti gIII protein antiserum (fig. 38). This analysis enables estimation of the amount of gIII fusion protein versus free gIII protein present on the phage(mid) particles.

Only a minute fraction of the gIII protein on the M13K07-rescued material is present as an intact fusion (fig 38). The fusion protein band is induced by IPTG, so is indisputably that synthesised by the phagemid. As expected, even when the lac promoter driving gIII fusion protein synthesis is fully induced (100µM IPTG), wild type gIII protein, at a lower copy number and driven from a far weaker promoter, predominates. This is in contrast to the pattern generated by the same clone rescued with M13K07 gIIIΔNo3, and the pattern generated by fd CAT2-scFv D1.3. In both of these latter cases, there is no competition with wild-type gIII and the fusion protein band is correspondingly stronger.

It is worthy of note that construction of M13K07 gIII Δ No3 was immensely inefficient: one clone from 20µg

of starting DNA. Moreover, the yield of gIII helper phage from overnight cultures is extremely low ca.10^6 cfu/ml compared with ca. 10^{11} cfu/ml for the parental phage. Despite this, M13K07 gIII No3 rescues the phagemid as well as the parental phage, as judged by the number of phagemid particles produced after overnight growth. This indicates that trans replication and packaging functions of the helper are intact and suggest that its own replication is defective. Hence it may be that inactivation of gIII is normally toxic to the host cell, and that M13K07 gIIIΔNo3 was isolated because of a compensating mutation affecting, for example, replication. Phage fd-tet is unusual in that it tolerates mutations in structural genes that are normally lethal to the host cell, since it has a replication defect that slows down accumulation of toxic phage products; M13K07 gIIIΔ No3 may also have such a defect.

M13K07g IIIΔ No 3 has been deposited at the National Collection of Type Cultures, 61 Colindale Avenue, London, NW9 6HT, UK (Accession No. NCTC 12478). On 28 June 1991, in accordance with the regulations of the Budapest Treaty. It contains a deletion of the M13 genome from bases 1979 to 2768 inclusive (see Van Wezenbeek, P.G.M.F. et al., Gene II p129-148, 1980 for the DNA sequence of the M13 genome).

Example 35

Selection of bacteriophage expressing scFv fragments directed against lysozyme from mixtures according to affinity using a panning procedure

For isolation of an antibody with a desired high affinity, it is necessary to be able to select an antibody with only a few fold higher affinity than the remainder of the population. This will be particularly important when an antibody with insufficient affinity has been isolated, for example, from a repertoire derived from an immunised animal, and random mutagenesis is used to prepare derivatives with potentially increased affinity. In this example, mixtures of phage expressing antibodies of different affinities directed against hen egg lysozyme were subjected to a panning procedure. It is demonstrated that phage antibodies give the ability to select for an antibody with a K_d of 2nM against one with a K_d of 13nM.

The oligonucleotides used in this example are shown in the list below:

OLIGONUCLEOTIDES

VHBHD13APA : 5'- CAC AGT GCA CAG GTC CAA CTG CAG GAG AGC GGT

VHFHD13 : 5'- CGG TGA CGA GGC TGC CTT GAC CCC

HD13BLIN : 5'- GGG GTC AGG GCA GCC TCG TCA CCG

HD13FLIN3 : 5'- TGG GCT CTG GGT CAT CTG GAT GTC CGA T

VKBHD13 : 5'- GAC ATC CAG ATG ACC CAG AGC CCA

VKFHD13NOT : 5'- GAG TCA TTC TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC
MURD13SEQ : 5'- GAG GAG ATT TTC CCT GT
HUMD13SEQ : 5'- TTG GAG CCT TAC CTG GC
FDPCRFOR : 5'- TAG CCC CCT TAT TAG CGT TTG CCA
FDPCRBAK : 5'- GCG ATG GGT GTT GTC ATT GTC GGC

Phage displaying scFv fragments directed against lysozyme were derived from cloned Fab fragments in plasmids.

Heavy and light chain variable regions were amplified by the polymerase chain reaction (PCR) from plasmids containing humanized VH-CH1 or VK-CK inserts suitable for production of Fab fragments (gift of J. Foote). The dissociation constant, Kd for different combinations of the two plasmids combined as Fabs, are shown below:

Heavy Chain Plasmid	Light Chain Plasmid	Kd
HuH-1	HuK-3	52 nM
HuH-1	HuK-4	180 nM
HuH-2	HuK-3	13 nM
HuH-2	HuK-4	(not determined)

Primary PCR

The primary PCR of the variable regions was performed by combining the following:

36.5 µl Water
5 µl PCR buffer (10x)
2 µl dNTP (5mM)
2.5 µl Back oligo (10 pmoles/µl) (VHBHD13APA or VKBHD13)
2.5 µl Forward oligo (10 pmoles/µl) (VHFHD13 or VKFHD13NOT)

The reaction is decontaminated by UV irradiation to destroy foreign DNA for 5 minutes, and 1 µl of plasmid DNA added (0.1 µg/µl). The pcr mixture was covered with 2 drops of paraffin oil, and placed on the pcr block at 94°C for 5 minutes before the addition of 0.5 µl of Taq DNA polymerase under the paraffin. The cycling conditions used were 94°C 1 min, 40°C 1 min, 72°C 1.5 min 17 cycles.

The linker $(Gly_4\text{-}Ser)_3$, was amplified from the anti-phOx (2-phenyloxazol-5-one) clone fd-CAT2-scFv NQ11, using the oligos HD13BLIN and HD13FLIN3, with 0.1µg of plasmid DNA. The PCR cycling used was 94°C 1 min, 25°C 1.5 min, for 17 cycles.

Amplified DNA was purified by running the samples on a 2% low melting point agarose gel at 90 mA, excising the appropriate bands and extracting the DNA using the Geneclean II Kit (BIO 101 Inc.) for the VH and VK, or by using Spin-X filter units (Costar) for the linker. A final volume of 10 µl was used to resuspend the extracted DNA.

PCR Assembly

Assembly of the four single chain Fv Humanized D1.3

(scFv HuD1.3) constructs was by the process of 'assembly by overlap extension' example 14.
The following were combined:
34.5 µl Water
5 µl PCR Buffer (10x)
2 µl dNTP (5 mM)
2.5 µl Back oligo (10 pmoles/µl) (VHBHD13APA)
2.5 µl Forward oligo (10 pmoles/µl) (VKFHD13NOT)

Once again, the reaction is decontaminate by UV treatment for 5 minutes before the addition of 1 µl of the primary PCR products; VH-1 or VH-2, VK-3 or VK-4, plus the linker DNA. The reaction was covered with 2 drops of paraffin, and heated at 94°C for 5 minutes before the addition of 0.5 µl of Taq Polymerase. The PCR cycling conditions used were 94°C 1 min, 60°C 1.5 min, 72°C 2.5 min for 20 cycles.

The aqueous layer under the paraffin was extracted once with phenol, once with phenol: chloroform, once with ether, ethanol precipitated, and resuspended in 36 µl of water. To this was added, 5 µl of 10x Buffer for NotI, 5 µl 1 mg/ml BSA, and 4 µl (40 U) of NotI (New England Biolabs). The restriction was incubated at 37°C overnight.

The DNA was ethanol precipitated and resuspended in 36 µl of water, and 5 µl 10x NEB Buffer 4, 5 µl 1 mg/ml BSA, and 2 µl (40 U) of ApaLI (New England Biolabs). This was incubated at 37°C for 5 hours; a further 2 µl of ApaLI was added and the reaction incubated at 37°C overnight.

The cut DNA was extracted by gel purification on a 1.3% low melting point agarose gel followed by treatment with Geneclean, to yield the insert DNA for cloning.

Vector fd CAT2 (prepared and digested with ApaLI and NotI as in example 20) and the scFv DNA were ligated as in example 20.

Analysis Of Clones

Colonies from the ligations were first screened for inserts by PCR screening. The PCR mixture was prepared in bulk by combining 14.8 µL 1x PCR Buffer, 1 µl dNTP (5 mM), 1 µl Back oligo (FDPCRBAK), 1 µl Forward oligo (FDPCRFOR), and 0.2 µl Taq polymerase per colony screened. 20 µl of this PCR mixture was aliquoted into a 96 well Techne plate. The top of a colony was touched with a toothpick and twirled quickly into the PCR mixture and the colony rescued by placing the toothpick in a Cellwell plate (Nunc) containing 250 µl of 2x TY medium. The PCR mixture is covered with 1 drop of paraffin and the plate placed on the block at 94°C for 10 minutes before cycling at 94°C 1 minute, 60°C 1 minute, 72°C 2.5 minutes.

The clones thus derived were named as below. The affinity of scFv fragments derived the Fab fragments was

not determined but previous results suggests that these are closely related although not necessarily identical (R.E. Bird & B.W. Walker TIBTECH 9 132-137, 1991).

Construct Name	Composition	Affinity of Fab (Kd)
TPB1	VH-HuH2-$(Gly_4\text{-}Ser)_3$-VK-HuK3	13 nM
TPB2	VH-HuH1-$(Gly_4\text{-}Ser)_3$-VK-HuK4	180 nM
TPB3	VH-HuH2-$(Gly_4\text{-}Ser)_3$-VK-HuK4	(Unknown)
TPB4	VH-HuH1-$(Gly_4\text{-}Ser)_3$-VK-HuK3	52 nM

Preparation of phage and ELISA was as described in example 6. The clones generated in fd CAT2 were shown to bind lysozyme as expected.

Affinity selection

Selection of Highest Affinity Binding Phage

Mixing experiments were performed in which fd-CAT2 scFvD1.3 phage (example 19) were mixed with either fd-CAT2 TPB1, fd-CAT2 TPB2, or fd-CAT2 TKPB4, and used in one round of panning.

The general method used for affinity selection by panning is that detailed below. Any deviation from this protocol is described at the relevant point. Panning plates were placed on a rocking platform between manipulations.

Falcon 35 mm Tissue Culture dishes were coated overnight with 1 ml of Lysozyme (various concentrations) dissolved in 50 mM Sodium Hydrogen Carbonate, pH 9.6, and blocked with 2 ml 2% MPBS at room temperature for 2 hours. Phage were prepared in 1 ml 2% MPBS and rocked at room temperature for 2 hours. Plates were washed for 5 minutes with 2 ml of the following solutions; 5 times with PBS, PBS-Tween, 50 mM Tris-HCl, pH 7.5; 500 mM Sodium Chloride, 50 mM Tris-HCl, pH 8.5; 500 mM Sodium Chloride, 50 mM Tris-HCl, pH 9.5; 500 mM Sodium Chloride, 50 mM Sodium Hydrogen Carbonated, pH 9.6; 500 mM Sodium Chloride. Phage were then eluted by adding 1 ml 100 mM Triethylamine and rocking for 5 minutes before removing the eluate which was neutralised with 100 µl 1.0 M Tris-HCl, pH 7.4.

Plates were coated overnight with Lysozyme at the concentration listed below.

Colonies from the single round of panning were probed with either MURDSEQ (for fdCAT2 scFvD1.3) or HUMD13SEQ (for fdCAT2 TPB constructs).

Circles of nitrocellulose (Schleicher & Schuell, BA 85, 0.45 µm) were labelled in pencil and lowered gently onto the colonies derived from the panning experiments and left for one minute. The filters were then pulled off quickly from one edge and placed colony side up on a piece of 3MM paper (Whatman) soaked in Denaturing

solution (500 mM Sodium Hydroxide; 1.5 M Sodium Chloride) for 5 minutes. They were then transferred to 3MM soaked in Neutralizing Solution (3.0 M Sodium Chloride; 500 mM Tris-HCl, pH 7.5) for 1 minute, and then to 3MM soaked in 5x SSC; 250 mM Ammonium Acetate for 1 minute. The filters were then air dried before baking in an 80°C vacuum oven for 30 minutes.

The oligonucleotide probe was prepared by combining the following:

2 µl oligonucleotide (1 pmoles/µl)
2 µl γ-32P ATP (3000 Ci/mmole) (Amersham International plc)
2 µl 10 x Kinase buffer (0.5 M Tris-HCl, pH 7.5; 100 mM Magnesium Chloride; 10 mM DTT)
12 µl Water
2 µl Polynucleotide Kinase (20 Units)
This was incubated at 37°C for 1 hour.

Hybridization was performed in the Techne HB-1 Hybridiser. The baked filters were pre-hybridized at 37°C in 40 ml of Hybridization Buffer (10 ml 100 mM Sodium pyrophosphate; 180 ml 5.0 M Sodium chloride; 20 ml 50x Denharts Solution; 90 ml 1.0 M Tris-HCl, pH 7.5; 24 ml 250 mM EDTA; 50 ml 10% NP40; made to 1 litre with water; 60.3 mg rATP; 200 mg yeast RNA (Sigma)), for 15 minutes before the addition of the 20 µl of the kinased oligo. The filters were incubated at 37°C for at least one hour, and then washed 3 times with 50 ml of 6x SSC at 37°C for 10 minutes (low stringency wash). Filters were air dried, covered with Saran wrap and exposed overnight with Kodak X-AR film.

Selection of fd-CAT2 scFv D1.3 from fd-CAT2 TPB4

Figure 39, summarizes the results from panning experiments using a mixture of the high affinity fd-CAT2 scFv D1.3 phage (Kd-2 nM) and the fd-CAT2 TPB4 construct (Kd-52 nM).

At a coating concentration of 3000 µg/ml Lysozyme, little or no enrichment could be obtained. It was however, possible to get enrichment for the scFv D1.3 phage when a lower concentration of Lysozyme was used for coating the plates. The best enrichment value obtained was from 1.5% fd-CAT2 scFv D1.3 in the starting mixture, to 33% fd-CAT2 scFv D1.3 in the eluted faction, on a plate coated overnight with 30 µg/ml Lysozyme.

Selection of fd-CAT2 scFv D1.3 from fd-CAT2 TPB1

Enrichment for the high affinity scFv D1.3 phage over the fd-CAT2 TPB1 phage (Kd-13) nM, could only be shown from experiments where the plates had been coated overnight with low concentrations of Lysozyme, as shown in Figure 40.

In summary, single chain Fv versions of a series of humanized D1.3 antibodies have been constructed in phage fd-CAT2. By affinity selection of fd-CAT2 phage

mixtures, by panning in small petri dishes, it was shown that the high affinity scFv D1.3 phage, could be preferentially selected for against a background of lower affinity scFv HuD1.3 phage.

Example 36

Expression of Catalytically Active Staphylococcal Nuclease on the Surface of Bacteriophage fd

Examples 11 and 12 showed that alkaline phosphatase from E.coli can be expressed as a catalytically active enzyme on the surface of bacteriophage fd. Here we show that Staphylococcal nuclease can also be expressed in a catalytically active form suggesting that this methodology may be general.

The gene for the enzyme Staphylococcal nuclease (SNase) was amplified from M13 mp18 - SNase (Neuberger, M.S. et al Nature 312 604-608, 1984) by PCR using primers with internal ApaLI (5'-GGAATTCGTGCACAGAGTGCAACTTCAACTAAAAAATTAC-3') and NotI (5'-GGGATCCGCGGCCGCTTGACCTGAATCAGCGTTGTCTTCG-3') restriction sites, cloned into phage vector fd-CAT2 after digestion with ApaLI-NotI restriction enzymes and the nucleotide sequence of the SNase gene and junctions with gene III checked by DNA sequencing. The fd-tet-SNase phage was prepared from the supernatant of infected E.coli TG1 cultures by three rounds of PEG precipitation, and the fusion protein demonstrated by SDS-gel electrophoresis and Western blotting using rabbit anti-g3p antiserum (Prof. I. Rasched, Konstanz) and peroxidase-labelled goat anti-rabbit antibodies (Sigma) (Fig.41) as described in example 27. As well as the fusion protein band (calculated Mr 59749, but runs at a higher position due to the aberrant g3p behaviour), a smaller (proteolytic ?) product is seen.

The fusion protein was shown to be catalytically active by incubation of the fd-tet-SNase phage (4 x 10^9 tetracyclin resistant colonies [TU]) with single stranded DNA (1 µg) for 1 hr at 37°C in the presence of Ca_2+, and analysis of the digest by agarose gel electrophoresis (Figure 42). Nuclease activity was not detected with the parent fd-CAT2 (2 x 10^{10} TU) phage alone or after three rounds of PEG precipitation of mixtures of fd-CAT2 (2 x 10^{10} TU) with SNase (0.7 µg). Thus the nuclease activity results from the display of the enzyme on the surface of the phage and not from co-precipitated or soluble SNase set free by degradation of the fusion protein. The nuclease activity of fd-tet-SNase (Figure 42) lies in the same order of magnitude, (2 x 10^8 TU and assuming three copies of SNase per TU) as an equimolar amount of SNase (0.03 ng or 10^9 particles), and like the authentic SNase was dependent on Ca^2+, since incubation with 40 mM $MgCl^2$ and 25 mM EGTA blocked activity (not shown).

Example 37: Display of the Two Aminoterminal Domains of Human CD4 on the Surface of fd Phage

The protein CD4, a member of the immunoglobulin superfamily, is a cell surface receptor involved in MHC class II restricted immune recognition. It is also recognised by the protein gp120 derived from the human immunodeficiency virus (AIDS virus). The first two domains (named V1 and V2, residues 1-178) of the surface antigen CD4 were amplified from pUC13-T4 (gift from T. Simon) containing the human cDNA of CD4, by PCR using primers with internal ApaLI (5'-GGA ATT CGT GCA CAG AAG AAA GTG GTG CTG GGC AAA AAA GGG G-3') and NotI (5'-GGG ATC CGC GGC CGC AGC TAG CAC CAC GAT GTC TAT TTT GAA CTC-3') restriction sites. After digestion with these two enzymes, the PCR-product was cloned into fdCAT2, and the complete nucleotide sequence of the CD4-V1V2 DNA and junctions with gene III checked by dideoxy sequencing using oligonucleotides fd-seq1 (5'-GAA TTT TCT GTA TGA GG), CD4-seq1 (5'-GAA GTT TCC TTG GTC CC-3') and CD4-seq2 (5'-ACT ACC AGG GGG GCT CT-3'). In the same way, a fd-CD4-V1 version was made, linking residues 1-107 to the N-terminus of gene III, using previously mentioned primers and oligonucleotide 5'-GGG ATC CGC GGC CGC GGT GTC AGA GTT GGC AGT CAA TCC GAA CAC-3' for amplification, PCR conditions and cloning were essentially as described in example 15 except that digestion was with ApaLI and NotI (used according to the manufacturers instructions).

Both fd-CD4-V1 and fd-CD4-V1V2 phages were prepared from the supernatant of infected E.coli TG1 cultures by three rounds of PEG precipitation, thereby concentrating the sample 100-fold for ELISA analysis. The fusion protein was detected in a Western blot (results not shown) with a rabbit anti-gene III antiserum, and revealed bands of the expected size.

Binding of the CD4 moiety to soluble gp120 (recombinant HIV-IIIB gp120 from CHO cells, ADP604, obtained from the Aids Directed Programme, National Institute for Biological Standards and Controls, South Mimms, Potters Bar, UK) was analysed in an ELISA, using 5 µg/ml gp120 for coating (overnight, in PBS). Anti-M13 antiserum was used to detect bound phage; all other conditions were as in Example 9. Figure 43 shows the ELISA signals of wild-type phage (fd-tet) and both CD4-phages. Both CD4-phages can bind gp120, but fd-CD4-V1V2 binds much stronger to gp120 than fd-CD4-V1. The binding competitors, soluble CD4 (recombinant soluble CD4 from Baculovirus, ADP 608; from the AIDS Directed Programme) (25 µg/ml) or soluble gp120 (20 µg/ml), added together with the 50 µl phage stock sample during the ELISA, decreased the signal to background level. These results indicate that phage binding to gp120 is mediated by the CD4 molecule displayed at its surface,, and that binding

is stronger when the two aminoterminal domains of CD4 are presented.

Thus, CD4 is a cell surface receptor molecule which is active when displayed on bacteriophage fd. Like the PDGF-BB receptor, the functional display of which is described in examples 15 and 16, CD4 is a member of the immunoglobulin superfamily and this result suggests that this class of molecule may be generally suitable for display on the surface of phage.

Example 38 Generation and Selection of Mutants of an Anti-4-hydroxy-3-nitrophenylacetic acid (NP) Antibody expressed on Phage using Mutator strains

It will sometimes be desirable to increase the diversity of a pool of genes cloned in phage, for example a pool of antibody genes, or to produce a large number of variants of a single cloned gene. There are many suitable in vitro mutagenesis methods. However, an attractive method, particularly for making a more diverse population of a library of antibody genes, is to use mutator strains. This has the advantage of generating very large numbers of mutants, essentially limited only by the number of phage that can be handled. The phage display system allows full advantage to be taken of this number to isolate improved or altered clones.

Nucleotide sequences encoding an antibody scFv fragment directed against 4-hydroxy-3-nitrophenylacetic acid (NP), scFvB18, derived as in example 14 from a monoclonal antibody against NP were cloned into fdCAT2 using ApaLI and NotI restriction sites as in example 11 to create fdCAT2scFvB18 or into fdDOGKan (fdCAT2 with its tetracycline resistance gene removed and replaced by a kanamycin resistance gene) using PstI and NotI restriction sites to create fdDOGKanscFvB18 or into the phagemid vector pHEN1 using the restriction sites SfiI and NotI as a fusion protein with gene III to create pHEN1scFvB18.

The following mutator strains (R. M. Schaaper & R.L. Dunn J. Mol. Biol. 262 1627-16270, 1987; R. M. Schaaper Proc. Natl. Acad. Sci. U.S.A. 85 8126-8130 1988) were used:

NR9232: ara, thi, mutD5-zaf13::Tn10, prolac, F'prolac
NR9670: ara, thi, azi, mutT1, leu::Tn10, prolac
NR9292: ara, thi, mutH101, prolac, F'prolac
NR9084: ara, thi, mutT1, azi, prolac, F'prolacI$^-$Z$^-\Delta$M15 M15
NR9046: ara, thi, supE, rif, nalA, metB, argE(am), prolac, F'prolac

were kind gifts of Dr. R. M. Schaaper (Department of Health & Human Services, N1H, PO Box 12233, Research Triangle Park, N.C. 27709)

NR9046mutD5: NR9046 mutD5::Tn10
NR9046mutT1: NR9046 mutT1::Tn10

were constructed by P1 transduction according to standard procedures. Mutator strains were transfected with fdCAT2scFvB18 of fdDOGKanscFvB18 and transfectants selected for antibiotic resistance. Transfectants were grown for 24h at 37°C before mutant phage was harvested by PEG precipitation. The mutant phage were selected on a 1ml NIP (4-hydroxy-3-iodo-5nitrophenylacetic acid)-BSA-Sepharose affinity column (prepared according to the manufacturers instructions) prewashed with 200ml of PBS and blocked by 20ml MPBS. Phage were loaded on the column in 10ml MPBS and unbound material reapplied to ensure complete binding. The column was subsequently washed with 10ml of MPBS and 500ml of PBS. Phage bound to the affinity matrix was eluted with 5 column volumes of 0.33 mM NIP-Cap (example 48).

Phage eluate was incubated for 30min to 1h with log phase ($2x10^8$ cells/ml) E.coli mutator strains without antibiotic selection. The infected cells were then diluted 1:100 in 2xTY and grown for 24h with antibiotic selection (15µg/ml tetracyclin or 30µg/ml kanamycin for fdCAT2scFvB18 or fdDOGKanscFvB18 respectively). Phage from this culture was used for another round of affinity selection and mutation.

Binding of phage antibodies was assayed by ELISA as in example 9 except that ELISA plates were coated with NIP-BSA (4-hydroxy-3-iodo-5-nitrophenylacetyl-BSA; 0.4 mg/ml). Culture supernatants were prepared following growth in Cellwells as described in example 21 and 20µl of culture supernatant was added to each well diluted to 200µl with MPBS.

Phage samples giving signals in ELISA of more than twice the background were tested ELISA as above for non-specific binding against lysozyme, BSA or Ox-BSA (example 9). Specificity for NIP was further confirmed by an ELISA in which serial dilutions of NIP-CAP were added together with phage antibodies. Addition of increasing concentrations of NIP-CAP reduced the ELISA signal to the background level.

Phage giving positive signals in ELISA were sequenced and 2 different mutants were subcloned into pHEN1 phagemid and transformed into HB2151 for soluble expression and TG1 for phage display (example 27).

For expression of soluble scFv fragments, transformants in E.coli HB2151 were grown at 37°C in 1 litre 2xTY, 0.2% glucoe, 0.1mg/ml ampicillin to an OD600 of 1 and expression of soluble scFv fragments induced by adding IPTG to 1mM. Cultures were shaken at 30°C for 16h.

Soluble scFvB18 was concentrated from crude bacterial supernatant in a FLOWGEN ultrafiltration unit to a volume of 200ml.

The concentrate was passed two times over a 2ml

column of NIP-BSA-Sepharose prewashed with 200ml of PBS. The column was washed with 500ml of PBS and 200ml of 0.1M Tris pH7.5, 0.5M NaCl and phage antibodies eluted with 50mM Citrate buffer pH2.3. The eluate was immediately neutralised with 1MTris pH8. The eluate was dialysed against two changes of 1 litre PBS, 0.2mM EDTA, Precipitated protein was removed by centrifugation at 10000g and protein yield was determined by measuring the absorbance at 280nm of the supernatant.

After 4 rounds of mutation and selection, isolated clones were screened and in one or two rare examples strongly positive ELISA signals were obtained from phage antibodies derived from the mutation of each of fdCAT2scFvB18 and fdDOGKanscFvB18 in the ELISA. The ELISA conditions were such that the parent phage fdCAT2scFvB18 only generated weak signals. These phage antibodies giving strongly positive ELISA signals were enriched in further rounds by a factor of roughly 2.5 per round. Forty phage antibodies giving strongly positive signals were sequenced and they each displayed single mutations in six different positions in the scFvB18 nucleotide sequences, five of which reside in the light chain. More than 70% of the mutations occurred at positions 724 and 725 changing the first glycine in the J segment of the light chain (framework 4) to serine (in 21 cases) or aspartate (in 3 cases). The mutations found are shown in Table 9. The sequence of scFvB18 is shown in Figure 44.

The nucleotide sequences encoding the scFv fragments of a framework mutant with the above glycine to serine mutation, as well as a mutant where Tyr in the CDR3 of the light chain had been mutated to aspartate, were amplified by PCR from the phage antibody clones and subcloned into pHEN1 phagemid (essentially as in example 25). This avoids possible problems with geneIII mutations caused by the mutator strains. The same pattern of ELISA signals was seen when the mutants were displayed on phage following rescue of the phagemid with helper phage (as described in example 25) as when the mutants were assayed when expressed from the phage genome as above.

The scFv fragments from scFvB18 and the scFv fragments containing the glycine to serine and tyrosine to aspartate mutations respectively were expressed in solution (following transformation into E.coli HB2151 as in example 27) at 30°C. They showed no differences in the ELISA signals between wild-type B18 and the framework mutant. The signal obtained from the phage antibody with the Tyr mutated to aspartate in CDR3 of scFvB18 was about 10x stronger. Expression yields were found to be comparable as judged by Western blotting using an antiserum raised against g3p (as described above).

Affinity measurements were performed using fluorescence quenching as described in example 23. Affinity measurement of affinity purified scFv fragments however showed scFvB18, and the scFvB18 (Gly->Ser) and scFvB18(Tyr->Asp) mutants all to have a comparable affinity of 20nM for NIP-CAP.

A Western blot using an anti-geneIII antibody showed the framework mutant had suffered significantly less proteolytic cleavage than scFvB18.

Hence, the use of mutator strains generates a diverse range of mutants in phage antibodies when they are used as hosts for clones for gene III fusions. In this case some of the clones exhibit higher ELISA signals probably due to increased stability to proteolyic attack. The mutator strains can therefore be used to introduce diversity into a clone or population of clones. This diversity should generate clones with desirable characteristics such as a higher affinity or specificity. Such clones may then be selected following display of the proteins on phage.

Example 39 Expression of a Fv Fragment on the Surface of Bacteriophage by Non-Covalent Association of VH and VL domains

This example shows that functional Fv fragments can be expressed on the surface of bacteriophage by non-covalent association of VH and VL domains. One chain is expressed as a gene III fusion and the other as a soluble polypeptide. Thus Fv fragments can be used for all the strategies discussed for Fab fragments including dual combinatorial libraries (example 26).

A useful genetic selection system for stably associated Fv fragments could be established if the expression of Fv fragments as fusion proteins on the phage surface would be possible such that one V domain is fused to the gene III protein and the other V domain is expressed separately in secreted form, allowing it to associate with the V domain on the fusion protein provided the interaction strength is sufficiently high. This idea was tested in a model experiment using the V domains from the anti-hen egg lysozyme antibody D1.3 by fusing the D1.3 VK gene to gene III and separately expressing the D1.3 VH domain.

Experimentally this was achieved as follows: The vector fd-DOG1 was digested with the restriction enzymes PstI and XhoI. From the Fv expression plasmid pSW1-VHD1.3-VKD1.3myc version 3/pUC119 (Ward et al., 1989 supra) a Pst 1/Xho I-digested restriction fragment was isolated that carries the VH domain coding sequence (terminated by 2 stop codons), a spacer region between VH and VK genes including a ribosome-binding site for expression of the VK gene, a pelB leader sequence, and, following in frame, the VK gene. This fragment was

cloned into the digested fd-DOG vector to generate the construct fd-tet Fv D1.3. As shown on the map in Fig.45, the dicistronic VH/VK-gene III operon is transcribed from the gene III promoter; secretion of the VH domain is achieved by the gene III protein leader, secretion of the VK-geneIII fusion protein by the pelB leader sequence. For control purposes a second construct with the name fd-tet Fv D1.3 (ΔS-Stuffer) was made by a similar route as described above: the VH used in this construct carries an insertion of a 200 bp fragment in the Sty I restriction site at the junction of VH CDR 3/FR4, thus interrupting the VH with several in frame stop codons. It is known from previous work that this insertion sufficiently disrupts the VH structure to abolish binding to the antigen lysozyme when expressed either as a soluble Fv or single-chain Fv fragment or as a single-chain Fv fragment on phage surface. This construct was used as a control. TG1 bacteria carrying either the fd-tet Fv D1.3, fd-tet Fv D1.3 (ΔS-Stuffer) or as single-chain wild-type control fd-tet scFv D1.3 plasmids were grown in liquid culture (medium 2xTY containing 15 µg/ml tetracycline) for 24h to produce phage particles in the supernatant. After removal of bacterial cells by centrifugation the phage titer in the supernatants was determined by re-infecting exponentially growing TG1 cells with dilutions of the supernatants and scoring tetracycline-resistants colonies after plating on tetracycline-plates. The infectious phage titers achieved were $1x10^{11}$ tetR transducing units/ml for the single-chain wild-type control fd-tet scFv D1.3 and $2x10^{10}$ tetR transducing units/ml for Fv phage constructs fd-tet Fv D1.3 and fd-tet Fv D1.3 (ΔS-Stuffer).

ELISA of hen egg lysozyme was performed as in example 2.

The results are shown in Fig.46. Phage derived from bacteria carrying and expressing the Fv construct fd-tet Fv D1.3 bind to the immobilised hen egg lysozyme, and when taking the phage titer into account, indeed apparently better than the single-chain Fv bearing phages produced by fd-tet scFv D1.3 carrying bacteria. The specificity of the reaction and the requirement for a functional VH domain is demonstrated by the fd-tet Fv D1.3 (ΔS-Stuffer) control in which disruption of the VH domain and consequently of the Fv fragment association eliminates binding to lysozyme.

As a final control of the expected structure of the VK/geneIII fusion protein a Western Blot was carried out. 20 µl of phage suspensions concentrated 100 fold by two sequential precipitations with PEG were applied to a 10% SDS-PAGE gel, electrophoretically separated and then transferred to a PVDF membrane (Immobilon, Millipore) in a semi-dry Western transfer apparatus (Hoefer). Remaining binding sites on the filter were blocked by 1h

incubation with 3% BSA in PBS, and detection of the gene III protein accomplished by incubation with a 1:1000 diluted rabbit anti-geneIII antiserum for 2h, several washes in PBS/0.1% Tween 20, incubation with peroxidase-conjugated goat anti-rat immunoglobulin antibodies, washes and development with the chromogenic substrate diaminobenzidine/$CoCl_2$/0.03% H_2O_2. The Fv phage fd-tet Fv D1.3 yields a band for the gene III fusion protein (data not shown), that is intermediate in size between the bands obtained for a wild-type gene III protein from fd-DOG1 and the scFv-gene III fusion protein from fd-tet scFv D1.3, thus proving the presence of a single immunoglobulin domain covalently fused to the gene III product int he Fv phage.

In summary, Fv-gene III fusions in which one V domain is fused to the gene III protein and the other V domain associates non-covalently can be presented in functionally active form on the surface of filamentous phage. This opens the possibility to genetically select for stably associated Fv fragments with defined binding specificities from V gene libraries expressed in phages.

Example 40 A PCR Based Technique for one step Cloning of Human V-genes as Fab Constructs

This example describes a PCR based technique to "assemble" human Fabs by splicing together the heavy and light chain DNA with a separate piece of 'linker' DNA. A mixture of universal primers is used which should make the technique applicable to all human V-genes.

The general technique for PCR assembly of human V-genes to create a Fab construct is described. The efficiency of this technique was assessed by "assembling", cloning and expressing a human anti rhesus-D (Rh-D) Fab from a IgG-K monoclonal hybridoma. We also demonstrate the potential to rescue human monoclonal antibodies from polyclonal cell populations by assembling, cloning, expressing and isolating an IgG-lambda monoclonal anti-Rh-D Fab from a polyclonal lymphoblastic cell line (LCL).

The overall strategy for the PCR assembly is shown in fig.47 and is described in more detail below. For Fab assembly, the VH-CH1 and VK-CK or V lambda-C lambda light chains are amplified from first strand cDNA and gel purified. Heavy and light chain DNA are then combined together with linker DNA and flanking oligonucleotides in a new PCR reaction. This results in a full length Fab construct since the 5' end of the linker DNA is complementary to the 3' end of the CH1 domain and the 3' end of the linker is complementary to the 5' end of the light chain domain. The linker DNA contains terminal residues of the human CH1 domain, the bacterial leader sequence (pelB) for the light chain and the initial residues of the VK or V lambda light chain (fig.2).

Finally, after gel purification, the Fab construct is reamplified with flanking oligonucleotides containing restriction sites for cloning.

Oligonucleotide primers: In order to develop the PCR cloning of human V genes it was necessary to design a new range of human specific oligonucleotide primers.

The PCR primers at the 5' end of the VH and VK and Vlambda gene exon (BACK primers) are based on sequence data extracted from the Kabat database, (Kabat, E.A. et al, Sequences of Proteins of Immunological Interest. 4th Edition. US Department of Health and Human Services. 1987) the EMBL database, the literature (Chuchana, P., et al, Eur J. Immunol. 1990. 20:1317) and unpublished data. The sequence of the VH, VK and Vlambda primers are given in table 1. In addition, extended VH primers with SfiI sites at the 5' end were also designed (Table 10) for adding a restriction site after assembly.

Table 10 also shows the 3' primers (FORWARD primers) designed for the PCR based cloning of human V genes. There are two sets of these depending on whether a Fab or scFv is to be produced. For Fab assembly, the forward primer was based at the 3' end of the CH1 domain, CK domain and Clambda domain. In addition, the CK and C2 FORWARD primers were also synthesized as extended versions with NotI sites at their 5' ends.

Primers complementary to the CH1 forward primers and the VkK and V lambda back primers were synthesized to permit generation of linker DNA by PCR amplification of a plasmid template containing the Fab linker (Table 10). To ensure adequate amplification, the primers were extended into the actual linker sequence.

A RNA preparation

This is essentially the same as described in Example 14, but using material of human origin. In the results given in this example human hybridoma and human polyclonal lymphoblastic cell lines were used.

B cDNA preparation

Approximately 4µg of total RNA in 20ul water was heated at 65°C for 3 minutes, quenched on ice and added to a 30 ul reaction mixture resulting in a 50ul reaction mixture containing 140mM KCl, 50mM Tris, HCl (pH8.1 @ 42°C), 8mM MgCl2, 10mM DTT, 500uM deoxythymidine triphosphate 500 uM deoxycytosine triphosphate, 500 uM deoxyadenosine triphosphate and 500 uM deoxyguanosine triphosphate, 80 units of human placental RNAse inhibitor and 10pmol of the appropriate Forward primer (HulgG1-4CH1FOR, HuIgMFOR, HuCKFOR, HuCLFOR). Two ul (50 units) of avian myeloblastosis virus (AMV) reverse transcriptase was added, the reaction incubated at 42°C for 1 hour, heated to 100°C for 3 minutes, quenched on ice and centrifuged for 5 minutes.

C Primary PCRs

For the primary PCR amplifications, an equimolar mixture of the appropriate family based BACK and FORWARD primers was used. (See specific examples 40a and 40b given later in this example). A 50ul reaction mixture was prepared containing 5ul of the supernatant from the cDNA synthesis, 20 pmol total concentration of the FORWARD primers, 250 uM dNTPs, 50mM KCl, 100mM Tris. HCl (pH 8.3), 1.5 mM MgCl2, 175ug/ml BSA and 1ul (5 units) Thermus aquaticus (Taq) DNA polymerase (Cetus, Emeryville, CA). The reaction mixture was overlaid with paraffin oil and subjected to 30 cycles of amplification using a Techne thermal cycler. The cycle was 94°C for 1 minute (denaturation), 57°C for 1 minute (annealing) and 72°C for 1 minute (extension). The product was analyzed by running 5ul on a 2% agarose gel. The remainder was extracted twice with ether, twice with phenol/chloroform, ethanol precipitated and resuspended in 50ul of H_2O.

D Preparation of linker

To make the Fab linker DNA, 13 separate PCR reactions were performed using HulgG1-4CH1FOR and each of the reverse VK or V lambda oligonucleotides. The template was approximately 1ng of pJM-1Fab D1.3 (fig.48) The PCR reaction reagents were as described above and the cycle was 94°:1 min, 45°:1min and 72°:1 min. The linkers were analyzed on a 4% agarose gel, purified on a 2% agarose gel, eluted from the gel on a Spin-X column and ethanol precipitated.

E Assembly PCRs

For PCR assembly of a human Fab approximately 1ug of a primary heavy chain amplification and 1ug of a primary light chain amplification were mixed with approximately 250ng of the appropriate linker DNA in a PCR reaction mixture without primers and cycled 7 times (94°: 2 min, 72°:2.5 min) to join the fragments. The reaction mixture was then amplified for 25 cycles (94°:1 mi, 68°-72°:1 min, 72°:2.5 min) after the addition of 20 pmol of the appropriate flanking BACK and FORWARD primers.

F Adding Restriction Sites

The assembled products were gel purified and reamplified for 25 cycles (94°:1 min, 55°:1 min, 72°: 25min) with the flanking oligonuceotides containing the appended restriction sites. PCR buffers and NTPs were as described previously.

Specific examples of PCR assembly of human immunoglobulin genes

a. PCR assembly of a Fab from a human hybridoma: the human monoclonal anti Rh-D cell lines Fog-1 (IgG-k) was derived from EBV transformation of the PBLs of a Rh-D negative blood donor immunized with Rh-D positive blood and has been previously described (Melamed, M.D., et al., J. Immunological Methods. 1987. 104:245) (Hughes-Jones N.C., et al., Biochem. J. 1990. 268:135) (Gorick, B.D. et

al., Vox. Sang. 1988. 55:165) Total RNA was prepared from approximately 10^7 hybridoma cells. First strand cDNA synthesis was performed as described above using the primers HulgG1-4CH1FOR and HuCKFOR. Primary PCRs were performed for the VH-CH1 using a mixture of the 6 HuVHBACK primers and HuIgG1-4CG1FOR and for the VK-CK using a mixture of the 6 HuVKBACK primers and HuCKFOR. A Fab construct was assembled as described above, restricted with SfiI and NotI, gel purified and ligated into pJM-1Fab D1.3 restricted with SfiI and NotI. The ligation mixture was used to transform competent E.coli E.M.G. cells. Ninety-six clones were toothpicked into media in microtitre plate wells, grown to mid-log phase at 30°C and then expression of the Fab was induced by heat shocking at 42°C for 30 min followed by growing for 4 hours at 37°C. The ninety-six clones were then screened for anti-Rh-D activity as described below.

b. assembly of human Fabs from a polyclonal (LCL): A polyclonal LCL "OG" was derived from EBV transformation of approximately 10^7 peripheral blood lymphocytes (PBLs) from a Rh-D negative donor immunized with Rh-D positive red blood cells. The cells were plated at a concentration of approximately 10^5 cells per well. Positive wells were identified by screening the cells harvested and then subcloned once. Typing of the well indicated that an IgG-lambda antibody was being produced. At this stage, total RNA was prepared from approximately 10^6 cells. First strand cDNA synthesis was performed as described above using the primers HulgG1-4CG1FOR and HuCLFOR. Primary PCRs were performed for the VH-CH1 using a mixture of the 6 HuVHBACK2 primers and HulgG1-4 CG1FOR and for the V lambda-C lambda using a mixture of the 7 HuV BACK primers and HuC FOR. Restriction, cloning and screening proceeded as described. To determine the diversity of the clones, the VH and V lambda genes of 15 clones were PCR amplified, restricted with the frequent cutting restriction enzyme BstN1 and analyzed on a 4% agarose gel (see example 20).

Assay for anti-Rh-D activity and demonstration of specificity: A 5% (vol/vol) suspension of either Rh-D positive (OR2R2) or Rh-D negative (Orr) erythrocytes in phosphate buffered saline (PBS, pH 7.3) were incubated with a papain solution for 10 min at 37°C. The erythrocytes were washed three times in PBS and a 1% (vol/vol) suspension of erythrocytes was made up in PBS supplemented with 1% (vol/vol) of bovine serum albumin (BSA). Fifty ul of a papain treated erythrocyte suspension and 50ul of phage supernatant were placed in the wells of round bottom microtitre plates and the plates were placed on a TItertek plate shaker for 2 min. After 15 min incubation at 37°C 100 ul of PBS/BSA was added to each well. The plates were centrifuged at 200 g

for 1 min and the supernatant was discarded. The erythrocytes were resuspended in the remaining PBS/BSA and the Fab fragments were crosslinked by addition of the 9E10 monoclonal antibody (50ul a 1ug/ml solution in PBS/BSA) directed against the myc peptide tag (Ward, E.S., et al., Nature 1989. supra). The plates were placed at room temperature (RT) until sedimentation had occurred. Agglutination of erthrocytes caused a diffuse button of erythrocytes and the results were evaluated macroscopically. Specificity was confirmed with a standard prepapainized (as above) panel of 9 erythrocyte suspensions in PBS (all suspensions blood group O, 4 D positive and 5 D negative) known to have homozygous expression of all the clinically relevant erythrocyte blood group alloantigens. The number of copies of the D antigen on the D positive cells varied between 10,000 and 20,000 per erythrocyte depending on the Rh genotype. Briefly, 50 ul phage supernatant in PBS supplemented with 2% (vol/vol) skimmed milk was mixed with 50 ul of a 2% erythrocyte suspension in PBS in glass tubes and incubated for 15 min at 37°C. After one wash with PBS/BSA the erythrocytes were pelleted and resuspended in 50 ul donkey anti-human lambda light chain (Sigma L9527, diluted 1:40 in PBS/BSA). The tubes were centrifuged for 1 min at 200g and agglutination was read macroscopically using "tip and roll" method.

Results

a PCR assembly of a Fab from a human hybridoma: A single band of the correct size was obtained after amplification. Thirty-eight of 96 clones (40%) screened specifically agglutinated Rh-D positive but not Rh-D negative red blood cells. The results demonstrate a high frequency of successful splicing in the assembly process and the potential of this technique for one step cloning of human hybridomas.

b Assembly of human Fabs from a polyclonal lymphoblastic cell line (LCL): Analysis of the diversity of the clones indicated that 3 different heavy chain families and 2 different light chains families were present. Five anti-Rh-D specific clones were identified out of 96 screened. The VH and Vλ chains had identical nucleotide sequences in each clone and were typical of anti-Rh-D V-genes (unpublished results). The results demonstrate the potential of this technique to assemble, clone and isolate human antibody fragments from polyclonal cell populations (see also section on isolation of specific binding activities from an 'unimmunized' human library (examples 42 and 43).

Example 41

Selection of Phage Displaying a Human Fab Fragment

directed against the Rhesus-D Antigen by binding to Cells displaying the Rhesus D Antigen on their Surface

A large number of important antigens are integral components of cell surface membranes, i.e. they are cell surface antigens. These include tumor specific antigens and red and white blood cell surface antigens. In many instances, it would be important to isolate antibodies against these antigens. For example, antibodies directed against the rhesus-D (Rh-D) antigen on red blood cells are used both diagnostically and therapeutically. Many of these antigens are difficult to purify and some, like Rh-D, are not biologically active when isolated from the membrane. Thus, it would be useful to be able to affinity purify antibody fragments displayed on the surface of bacteriophage directly on cell surface antigens. To test the feasibility of affinity purification on cell surface antigens, the anti-Rh-D human monoclonal antibody Fog-B was displayed as a Fab fragment on the surface of bacteriophage fd. The displayed Fog-B Fab fragment bound antigen as determined by agglutination assay and could be affinity purified on the basis of its binding on the surface of Rh-D positive red blood cells but not Rh-D negative red blood cells.

Materials and Methods

Construction of a clone encoding an anti-Rh-D Fab fragment in phagemid pHENI and display of the Fab fragment on the surface of bacteriophage fd.

The human hybridoma Fog-B has been previously described (N.C. Hughes-Jones et al Biochem, J. 268 135 (1990). It produces an IgG-1/lambda antibody which binds the Rh-D antigen. RNA was prepared from 10^7 hybridoma cells using a modified method of Cathala (as described in example 14) and 1st strand cDNA synthesized using specific immunoglobulin heavy and light chain primers (HuVH1FOR [example 40] and HuCλ FOR (5'-GGA ATT CTT ATG AAG ATT CTG TAG GGG CCA C-3')) as described in example 14. The VH gene was subsequently amplified from an aliquot of the 1st strand cDNA using HuVH4aBACK and HuVH1FOR. The Vλ gene was amplified using a Vλprimer specific for Fog-B (VλFog-B, 5'-AAC CAG CCA TGG CC AGT CTG TGT TGA CGC AGC C-3'). The PCR conditions were as described in example 40. The PCR products were analyzed by running 5µl on a 2% agarose gel. The remainder was extracted twice with ether, twice with phenol/chloroform, ethanol precipitated and resuspended in 50µl of H_2O. The amplified VH DNA was digested with PstI and BstEII, and the amplified Vλ-Cλ DNA with NcoI and EcoR1. The fragments were purified on a 2% agarose gel, extracted using Geneclean, and sequentially ligated into the soluble expression vector pJM-1 Fab D1.3 (Fig 48). Clones containing the correct insert were initially identified by restriction analysis and verified by assay

of expressed soluble Fab (see example 23 for induction conditions). The Fog-B Fab cassette was amplified from pJM-1 by PCR using HuVH4BACK-Sfi and Hu Cλ-Not, digested with the appropriate restriction enzymes and ligated into pHEN1. Clones containing the correct insert were identified initially by restriction analysis and subsequently by assay (see example 25 for induction conditions).

Assay for soluble Fog-B Fab fragment and phage displayed Fog-B Fab fragment for anti-Rh-D activity and documentation of specificity.

Assay of the soluble expressed Fab was performed on unconcentrated E.coli supernatant. Assay of Fog-B displayed on the phage surface was performed on phage that had been concentrated 10 fold by PEG precipitation and then resuspended in PBS. the assays for activity and specificity are as described in example.

Cell surface affinity purification of phage displaying Fog-B anti-Rh-D Fab fragment

Purified Fog-B phage was mixed with purified phage Fd-Tet CAT-1 displaying the anti-lysozyme scFv D1.3 (pAbD1.3) in a ratio of approximately 1 Fog-B:50 scFvD1.3. Prepapainized erythrocytes (OR2R2 [Rhesus positive] or Orr [Rhesus negative]) were suspended in PBS supplemented with 2% skimmed milk powder in a concentration of 4x107/ml. One ml of this suspension was mixed with 10^{11} phage suspended in 2 ml of PBS supplemented with 2% skimmed milk and incubated for 30 min at room temperature under continuous rotation. The erythrocytes were washed three times with an excess of ice-cold PBS (10 ml per wash) and subsequently pelleted. The phage were eluted from the cells by resuspending in 200 µl of 76 mM citric acid pH 2.8 in PBS for 1 min. The cells were then pelleted by centrifugation for 1 min at 3000 rpm and the supernatant containing the eluted phage was neutralized by adding 200 µl of 240 mM Tris-base, 22mM Disodium hydrogen phosphate in 1% w/vol albumin. Serial dilutions of the eluate was used to infect TG1 cells. Fog-B Fab phage were selected on ampicillin plates and scFvD1.3 phage on tetracycline plates and the titre of each determined prior to selection, after selection on rhesus-D negative cells and after selection on rhesus-D positive cells.

Results

Fog-B Fab fragment displayed on the surface of the phage derived from the phagemid pHEN clone specifically agglutinated rhesus-D positive but not rhesus D-negative red blood cells. Affinity purification of the Fog-1 Fab phagemid on Rh-D positive red blood cells resulted in an enrichment from 1:50 to 1500:1 (Fog-B Fab:scFvD1.3), whereas purification on Rh-D negative red blood cells demonstrated essentially no enrichment (10 fold).

	TITRE		RATIO
	Fog-B Fab	scFvD1.3	Fog-B FAb/scFvD1.3
Prior to selection	1.0×10^8	5.0×10^9	1:50
Selection on Rh-D negative cells	2.0×10^4	1.0×10^5	1:5
Selection on Rh-D positive cells	6.0×10^6	4.0×10^3	1500:1

Example 42 A PCR Based Technique for One Step Cloning of Human scFv Constructs

Assembly of human scFv is similar to the assembly of mouse scFvs described in example 14. To develop the PCR cloning of human V genes it was necessary to design a new range of human specific oligonucleotide primers (table 10). The use of these primers for the generation of human Fabs is described in example 40. The assembly of human scFvs is essentially the same but requires a set of FORWARD primers complementary to the J segments of the VH, VK and V lambda genes. (For Fabs FORWARD primers complementary to the constant region are used.) The J segment specific primers were designed based on the published JH, JK and J lambda sequences (Kabat, E.A. et al, Sequences of Proteins of Immunological Interest. 4th Edition. US Department of Health and Human Services. 1987).

In addition, a different linker is needed for scFvs than for Fabs so for human scFvs a new set of primers was needed to prepare the linker. Primers complementary to the JH forward primers and the VK and V lambda back primers were synthesized to permit generation of linker DNA by PCR amplification of a plasmid template containing the scFv linker (Table 10, Fig. 49). To ensure adequate amplification, the primers were extended into the actual linker sequence. Using these primers to make the scFv linker DNA, 52 separate PCR reactions were performed using each of the 4 reverse JH primers in combination with each of the 13 reverse VK and V lambda oligonucleotides. The template was approximately 1ng of pSW2scD1.3 (Ward, E.S. 1989 supra) containing the short peptide (Gly4Ser)3 (Huston, J.S. et al., Gene 1989. 77:61)

A specific example of PCR assembly of a human scFv library

This example describes the generation of a human library of scFvs made from an unimmunized human:

500ml of blood, containing approximately 10^8 B-cells, was obtained from a healthy volunteer blood donor. The white cells were separated on Ficoll and RNA was prepared as described in example 14.

Twenty percent of the RNA, containing the genetic

material from approximately 2 x 10^7 B-cells, was used for cDNA preparation as described in example 40. Heavy chains originating from IgG and IgM antibodies were kept separate by priming cDNA synthesis with either an IgG specific primer (HuIgG1-4CH1FOR) or an IgM specific primer (HuIgMFOR). Aliquots of the cDNA was used to generate four separate scFv libraries (IgG-K, IgG-lambda, IgM-K and IgM-lambda) as described in example 40. The resulting libraries were purified on 1.5% agarose, electroeluted and ethanol precipitated. For subsequent cloning, the K and lambda libraries were combined giving separate IgG and IgM libraries.

Cloning of the library: The purified scFv fragments (1-4ug) were digested with the restriction enzymes NotI and either SfiI or NcoI. After digestion, the fragments were extracted with phenol/chloroform, ethanol precipitated. The digested fragments were ligated into either SfiI-NotI or NcoI-NotI digested, agarose gel electrophoresis purified pHEN1 DNA (6ug) (see example 24), in a 100 µl ligation mix with 2,000 U T4 DNA ligase (New England Biolabs) overnight at room temperature. The ligation mix was purified by phenol extraction and ethanol precipitated. The ligated DNA was resuspended in 10 µl of water, and 2.5 µl samples were electroporated into E.coli TG1 (50 µl). Cells were grown in 1 ml SOC for 1 hr and then plated on 2 x TY medium with 100 µg/ml ampicillin and 1% glucose (AMP-GLU), in 243 x 243 mm dishes (Nunc). After overnight growth colonies were scraped off the plates into 10 ml 2 x TY containing AMP-GLU and 15% glycerol for storage at -70°C as a library stock.

Cloning into SfiI-NotI and NcoI-NotI digested pHEN1 yielded libraries of 10^7 and 2 x 10^7 clones respectively for the IgM libraries and approximately 5 x 10^7 clones for each of the two IgG libraries.

Example 43 Isolation of binding activities from a library of scFvs from an unimmunized human

The ability to select binding activities from human antibody libraries displayed on the surface of phage should prove even more important than isolation of binding activities from murine libraries. This is because the standard way of generating antibodies via hybridoma technology has not had the success with human antibodies that has been achieved with mouse. While in some instances it will be possible to make libraries from immunized humans, in many cases, it will not prove possible to immunize due to toxicity or lack of availability of an appropriate immunogen or ethical considerations. Alternatively, binding activities could be isolated from libraries made from individuals with diseases in which therapeutic antibodies are generated by

the immune response. However, in many cases, the antibody producing cells will be located in the spleen and not available in the circulating pool of peripheral blood lymphocytes (the most easily accessible material for generating the library). In addition, in diseases associated with immunosuppression, therapeutic antibodies may not be produced.

An alternative approach would be to isolate binding activities from a library made from an unimmunized individual. This approach is based on estimates that a primary repertoire of 10^7 different antibodies is likely to recognize over 99% of epitopes with an affinity constant of 10^5 M^{-1} or better. (Pewrelson, A.S. Immunol. Rev, (1989) 110:5). While this may not produce high affinity antibodies, affinity could be boosted by mutation of the V-genes and/or by using the isolated VH domain in a hierarchical approach with a library of light chains (or vice versa). In this section,, we demonstrate the feasibility of this approach by isolating specific antigen binding activities against three different antigens from a library of scFvs from an unimmunized human.

Materials and Methods

The generation of the human scFv library used for the isolation of binding activities described in this example is detailed in example 42.

Estimation of diversity of original and selected libraries: Recombinant clones were screened before and after selection by PCR (example 20) with primers LMB3 (which sits 5' of the pelB leader sequence and is identical to the reverse sequencing primer (-40 n) of pUC19) and fd-SEQ1 (see example 37) followed by digestion with the frequent-cutting enzyme BstN1. Analysis of 48 clones from each unselected library indicated that 90% of the clones had inset, and the libraries appeared to be extremely diverse as judged by the BstNI restriction pattern.

Rescue of Phagemid libraries for enrichment experiments: To rescue phagemid particles from the library, 100 ml 2 x TY containing AMP-GLU (see example 42) was inoculated with 10^9 bacteria taken from the library (prepared in example 42) (approx. 10 µl) and grown for 1.5 hr, shaking at 37°C. Cells were spun down (IEC- centrifuge, 4 K, 15 min) and resuspended in 100 ml prewarmed (37°C) 2 x TY-AMP (see example 41) medium, 2 x 10^{10} pfu of VCS-M13 (Stratagene) particles added and incubated 30 min at 37° without shaking. Cells were then transferred to 900 ml 2 x TY containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) (AMP-KAN), and grown overnight, while shaking at 37°C. Phage particles were purified and concentrated by three PEG-precipitations (see materials and methods) and resuspended in PBS to 10^{13} TU/ml (ampicillin resistant

clones).

Enrichment for phOx:BSA binders by selection on tubes: For enrichment, a 75 x 12 mm Nunc-immunotube (Maxisorp; Cat. No. 4-44202) was coated with 4 ml phOx:BSA (1 mg/ml; 14 phOx per BSA in 50 mM NaHCO3 pH 9.6 buffer) overnight at room temperature. After washing three times with PBS, the tube was incubated for 2 hr at 37°C with PBS containing 2% Marvel (2% MPBS) for blocking. Following three PBS washes, phagemid particles (10^{13} TU) in 4 ml of 2% MPBS were added, incubated 30 min at room temperature on a rotating turntable and left for a further 1.5 hours. Tubes were then washed with 20 washes of PBS, 0.1% Tween 20 and 20 washes PBS (each washing step was performed by pouring buffer in and out immediately). Bound phage particles were eluted from the tube by adding 1 ml 100 mM triethylamine pH 11.5 and rotating for 15 min. The eluted material was immediately neutralised by adding 0.5 ml 1.0 M Tris-HCl, pH 7.4 and vortexed. Phage was stored at 4°C.

Eluted phage (in 1.5 ml) was used to infect 8 ml logarithmic growing E.coli TG1 cells in 15-ml 2 x TY medium, and plated on AMP-GLU plates as above yielding on average 10^7 phage infected colonies.

For selection of phOx:BSA binders, the rescue-tube enrichment -plating cycle was repeated 4 times, after which phagemid clones were analysed for binding by ELISA.

Enrichment for lysozyme binders by panning and on columns: A petri dish (35 x 10 mm Falcon 3001 Tissue culture dish) was used for enrichment by panning. During all steps, the plates were rocked on an A600 rocking plate (Raven Scientific). Plates were coated overnight with 1 ml turkey egg white lysozyme (3 mg/ml) in 50 mM sodium hydrogen carbonate (pH 9.6), washed three times with 2 ml PBS, and blocked with 2 ml 2% MPBS at room temperature for 2 hours. After three PBS washes approximately 10^{12} TU phage particles in 1 ml 2% MPBS were added per plate, and left rocking for 2 hr at room temperature. Plates were washed for 5 min with 2 ml of the following solutions: 5 times PBS, PBS-Tween (0.02% Tween-20), 50 mM Tris-HCl (pH 7.5) + 500 mM NaCl, 50 mM Tris-HCl (pH 8.5) + 500 mM NaCl, 500 mM Tris-HCl (pH 9.5) + 500 mM NaCl and finally 50 mM sodium hydrogen carbonate pH 9.6 Bound phage particles were then eluted by adding 1 ml 100 mM triethylamine pH 11.5 and rocking for 5 min before neutralising with 1 M Tris-HCl (pH 7.4) (as above). Alternatively, 1 ml turkey egg white lysozyme-Sepharose columns were used for affinity purification (McCafferty, J., et al., Nature 1990. 348: 552) Columns were washed extensively with PBS, blocked with 15 ml 2% MPBS, and phage (10^{12} TU) in 1 ml 2% MPBS loaded. After washing with 50 ml PBS, 10 ml PBS- Tween (PBS + 0.02% Tween-20), 5 ml of 50 mM Tris-HCl (pH 7.5) + 500 mM NaCl,

5 mM Tris-HCl 9pH 8.5) + 500 mM NaCl, 5ml of 50 mM Tris-HCl (pH 9.5) + 500 mM NaCl and finally 5 ml of 50 mM sodium hydrogen carbonate pH 9.6. Bound phage was eluted using 1.5 ml 100 mM triethylamine and neutralised with 1 M Tris-HCl (pH 7.4).

For selection of turkey egg white lysozyme binders, the rescue-tube enrichment-plating cycle or rescue-column-plating cycle was repeated 4 times, after which phagemid clones were analysed for binding by ELISA.

Rescue of individual phagemid clones for ELISA: Clones resulting from reinfected and plated phage particles eluted after 4 rounds of enrichment, were inoculated into 150 µl of 2 x TY-AMP-GLU in 96-well plates (cell wells, Nunclon), grown with shaking (250rpm) overnight at 37°C. A 96-well plate replicator ('plunger') was used to inoculate approximately 4 µl of the overnight cultures on the master plate into 200 µl fresh 2 x TY-AMP-GLU. After 1 hr, 50 µl 2 x TY-AMP-GLU containing 10^8 pfu of VCS-M13 was added to each well, and the plate incubated at 37°C for 45 min, followed by shaking the plate at 37°C for 1 hr. Glucose was then removed by spinning down the cells (4K, 15 min), and aspirating the supernatant with a drawn out glass pasteur pipet. Cells were resuspended in 200 µl 2 x TY-AMP-KAN (Kanamycin 50 ug/ml) and grown 20 hr, shaking 37°C. Unconcentrated supernatant containing phage was taken for analysis by ELISA.

ELISA

Analysis for binding to phOx:BSA, BSA or lysozyme was performed by ELISA (see example 9), with 100 µg/ml phOx:BSA or BSA, or 3 mg/ml turkey egg white lysozyme used for coating. Determination of cross reactivity to unrelated antigens with the isolated clones was also determined by ELISA on plates coated with 100 ug/ml of an irrelevant antigen (keyhole limpet haemocyanin (KLH), ovalbumin, chymotrypsinogen, cytochrome C, thyroglobulin, GAP-DH (glyceraldehyde-3-phosphate dehydrogenase), or trypsin inhibitor).

Characterization of ELISA positive clones: All antigen specific clones isolated were checked for cross reactivity against a panel of irrelevant antigens as described above. The diversity of the clones was determined by PCR screening as described above and at least two clones from each restriction pattern were sequenced by the dideoxy chain termination method.

Results

Isolation and characterization of phOx:BSA binders: After 4 rounds of selection, ELISA-positive clones were isolated for phOx:BSA. All clones originated from the IgM library. Of 96 clones analysed, 43 clones were binding to both phOx:BSA and BSA, with ODs ranging from 0.4 to 1.3 (background 0.125). These clones are

designated as BSA binders. The binding to BSA seemed to be specific, since none of the 11 clones analysed gave a signal above background when used in an ELISA with KLH, ovalbumin, chymotrypsinogen, cytochrome C, lysozyme, thyroglobulin, GAP-DH, or trypsin inhibitor. All BSA binding clones had the same BstNI restriction pattern, and 14 clones were completely sequenced. Thirteen of the fourteen clones had the same sequence, the VH was derived from a human VH3 family gene and the VL from a human V lambda 3 family gene (Table 1). The other BSA binder was derived from a human VH4 family gene and a human Vk1 family gene (data not shown).

One clone was isolated which bound to phOx:BSA only (OD 0.3), and bound phage could be completed off completely by adding 0.02 mM 4-ϵ-amino-caproic acid methylene 2-phenyl-oxazol-5-one (phOx-CAP) as a competitor. Also no binding above background could be detected to the panel of irrelevant proteins described above. The sequence revealed a VH derived from a human VH1 family gene and a VL derived from a human V lambda 1 family gene (Table 11).

Isolation and characterisation of lysozyme binders: After 4 rounds of selection, 50 ELISA-positive clones were isolated for turkey lysozyme. The majority of the clones, greater than 95%, were from the IgM library. The binding to lysozyme seemed to be specific, since none of the clones analysed gave a signal above background when used in an ELISA with KLH, ovalbumin, chymotrypsinogen, cytochrome C, thyroglobulin, GAP-DH, or trypsin inhibitor. The lysozyme binding clones gave 3 different BstNI restriction patterns, and at least 2 clones from each restriction pattern were completely sequenced. The sequences indicated the presence of 4 unique human VH-VL combinations. (Table 11).

Conclusion

The results indicate that antigen binding activities can be isolated from repertoires of scFvs prepared from IgM cDNA from human volunteers that have not been specifically immunized.

Example 44

Rescue of human IgM library using helper phage lacking gene 3 (δg3)

This example describes the rescue of gene 3 fusions from a human library using a helper phage with a gene 3 deletion.

100 µl of bacterial stock of the IgM phagemid library prepared as described (example 42), containing $5x10^8$ bacteria, was used to inoculate 100mls of 2xTY medium containing 100µg/ml ampicillin, 2% glucose (TY/Amp/Glu). This was grown at 37°C for 2.5 hours. 10 mls of this culture was added to 90 mls of prewarmed TY/Amp/Glu and infection carried out by adding 10mls of a

200 fold concentrate of KO7 helper phage lacking gene 3 (M13KO7gIIIΔNo.3) (example 34) and incubating for 1 hour at 37°C without shaking. Preparation of M13KO7gIII No.3 was as described in example 34. After centrifugation at 4,000 r.p.m. for 10 minutes the bacteria were resuspended in 100 mls of 2 x TY medium containing 100 µg/ml ampicillin (with no glucose). Titration of the culture at this point revealed that there were $1.9x10^8$ infected bacteria as judged by their ability to grow on plates containing both ampicillin (100µg/ml) and kanamycin (50µg/ml). Incubation was continued for 1 hour with shaking before transferring to 2.5 litres of 2xTY medium containing 100µg/ml ampicillin, 50µg/ml kanamycin, contained in five 2.5 litre flasks. This culture was incubated for 16 hours and the supernatant prepared by centrifugation. (10-15 minutes at 10,000 r.p.m. in a Sorvall RC5B centrifuge at 4°C). Phage particles were harvested by adding 1/5th volume of 20% polyethylene glycol, 2.5 M-NaCl, standing at 4°C for 30 minutes and centrifuging as above. The resulting pellet was resuspended in 40mls of 10mM Tris, 0.1mM EDTA pH 7.4 and bacterial debris removed by centrifugation as above. The packaged phagemid preparation was then re-precipitated, collected as above and resuspended in 10mls of 10mM Tris, 0.1mM EDTA pH 7.4. The litre of this preparation was $4.1x10^{13}$ transducing units/ml (ampicillin resistance).

Tubes coated with OX-BSA were prepared as described in example 45 for panning the phagemid library from example 42. The rescued library was also panned against tubes coated with bovine thyroglobulin (Sigma). These were coated at a concentration of 1mg/ml thyroglobulin in 50mM NaHCO3 pH9.6 at 37°C, overnight. Tubes were blocked with PBS containing 2% milk powder (PBS/M) and incubated with 1ml of the rescued phagemid library (the equivalent of 250mls of culture supernatant) mixed with 3mls of PBS/M for 3 hours. Washing, elution, neutralisation and infection were as described in example 45.

Results: Panning against oxazalone - BSA

The first round of panning against OX-BSA yielded $2.8x10^6$ phage. A large bacterial plate with $1.4x10^6$ colonies derived from this eluate was scraped into 10mls of 2xxTY, 20% glycerol, shaken for 10 minutes, aliquoted and stored. This was also used to inoculate a fresh culture for rescue with M13KO7gIII No.3. (Bacteria and rescued phage derived from first round panning against OX-BSA are named OXPAN1. Bacteria or rescued phage derived from second and third round pannings are named OXPAN2 and OXPAN3 respectively) Rescue of phagemid with M13KO7gIII No.3 after each round of panning was essentially as described above but using 5ml volumes for the initial cultures in TY/Amp/Glu, using 1ml of helper phage and transferring to 100-500mls of 2xTY medium

containing 100µg/ml ampicillin, 50µg/ml kanamycin. Second and third round panning steps were as described above for the first round, but using 0.8-1.0mls of 100 fold concentrated phage (the equivalent of 80-100 mls of culture supernatant). The eluate from the second round panning contained $8x10^8$ infectious particles and the eluate from the third round panning contained $3.3x10^9$ infectious particles.

Panning against thyroglobulin

The first round panning against thyroglobulin yielded $2.52x10^5$ infectious particles. Half of the eluate was used to generate $1.26x10^5$ bacterial colonies on a large plate. These colonies were scraped into 10mls of 2xTY, 20% glycerol, shaken for 10 minutes, aliquoted and stored. These bacteria and rescued phage derived from them are termed THYPAN1, and used to inoculate a fresh culture for rescue with M13KO7gIII No.3 to give a polyclonal rescued phage preparation. Material similarly derived from second and third round pannings are termed THYPAN2 and THYPAN3 respectively. Second and their round pannings with thyroglobulin were as described for second and third round OX-BSA panning. The eluate from the second round panning contained $8x10^7$ transducing units and the eluate from the third round panning contained $6x10^7$ infectious particles.

ELISA screening of clones derived by panning

40 colonies derived form the third round of panning against thyroglobulin (THYPAN3) were picked into a 96 well plate and grown overnight at 37°C in 200ul of TY/Amp/Glu. Similarly 48 colonies from two rounds and 48 colonies from three rounds of panning against OX-BSA were grown (OX-PAN2 and OX-PAN3). Polyclonal phage were prepared at the same time. Next day 5µl from each culture was transferred to 100µl of fresh prewarmed TY/Amp/Glu grown for 1.5 hours and M13KO7gIII No.3 added (2×10^5 infectious phage per well in 100µl of TY/Amp/Glu). these were incubated for 1 hour at 37°C without shaking, centrifuged at 4,000 r.p.m. for 10 minutes, resuspended in 150µl of 2xTY medium containing 100µg/ml ampicillin and incubated for a further hour with shaking before adding to 2mls of medium containing 100µg/ml ampicillin, 50µg/ml kanamycin. After overnight growth the cultures were centrifuged at 4,000 r.p.m. for 10 minutes and the supernatants collected. ELISA plates used to screen THYPAN3 clones were coated at 37°C overnight with 200µg/ml thyroglobulin in 50mM NaHCO3pH9.6. Plates used for OXPAN2 and OXPAN3 were coated at 100µg/ml OX-BSA in PBS at 37°C overnight.

120µl of culture supernatant was mixed with 30ul of 5x PBS, 10% milk powder and incubated at room temperature for 2 hours at room temperature. ELISAs were carried out as described in example 18.

For thyroglobulin, 18 out of 40 clones were positive (0.3-2.0 O.D. after 30 minutes). (A phage control (vector pCAT3) gave a reading of 0.07 O.D.). In addition, positives were also seen on the polyclonal phage preparations THYPAN1 (0.314 O.D.) and THYPAN2 (0.189 O.D.) compared with phage derived from the original non-panned phagemid library (0.069 O.D.). All polyclonal phage were PEG precipitated and used at a 10 fold concentration.

PCR reactions and BstN1 digests were carried out on the positive clones as described above and six different patterns of DNA fragments were obtained showing that at least six different clones had been isolated.

For OX-BSA after two rounds of panning, 30 of 48 clones were positive by ELISA and after three rounds , 42 of 48 were positive. In a separate experiment, positive signal was obtained from the polyclonal phage preparations OXPAN1 (0.988 OD) and OXPAN2 (1.717 OD) compared with phage derived from the original non-panned phagemid library (0.186 O.D.) after 30 minutes.

Specificity of clones for thyroglobulin or OX-BSA

Selected clones (11 anti-thyroglobulin, 5 anti-OX-BSA) representing each of the different BstNI restriction digest patterns were assayed for binding to a panel of irrelevant antigens. ELISA plates were coated with antigen (100 µl/ml in 50 mM $NaHCO_3$, pH 9.6) by overnight incubation at 37°C. The panel of antigens consisted of keyhole limpet haemocyanin, hen egg lysozyme, bovine serum albumin,ovalbumin, cytochrome c, chymotrysinogen, trypsin inhibitor, GAP-D11 (glyceraldehyde-3-phosphate dehydrogenase), bovine thyroglobulin and oxazolone-BSA. Duplicate samples of phage supernatant (80 µl + 20 µl 5 x PBS, 10% milk powder) were added to each antigen and incubated for 1 hour at room temperature. the ELISA was carried out as described in example 18.

Each of the thyroglobulin specific clones (11 from 11) were positive for thyroglobulin (OD 0.12 - 0.76) but after 60 minutes showed no binding (OD<0.03) to any of the 9 irrelevant antigens. Similarly of the 5 OX-BSA specific clones 3 had an OD 0.07 - 0.52 compared to ODs < 0.02 for the irrelevant antigens. None of the 5 clones had any binding to BSA alone.

Thus positive clones can be isolated after only two rounds of panning by rescuing with M13KO7gIII No.3. In addition there is a greater likelihood with this helper of generating phage particles with more than one intact antibody molecule. This will potentially increase the avidity of phage-antibodies and may enable isolation of clones of weaker affinity.

Example 45: Alteration of fine specificity of scFv D1.3 displayed on phage by mutagenesis and selection on immobilised turkey lysozyme

The D1.3 antibody binds hen egg lysozyme (HEL) with an affinity constant of 4.5 x $10^7 M^{-1}$ whereas it binds turkey egg lysozyme (TEL) with an affinity of $<1x10^5 M^{-1}$. (Harper et al (1987) Molecular Immunology 24 p97-108, Amit et al (1986) Science 233 p747-753).

It has been suggested that this is because the glutamine residue present at position 121 of HEL (gln121) is representated by histidine residue at the same position in TEL. Thus mutagenising the D1.3 antibody residues which interact with gln121 of HEL may facilitate binding to TEL.

According to Amit et al, supra, tyrosine at amino acid position 32, phenylalanine at position 91 and tryptophan at position 92 of the light chain interact with gln121 of HEL. In addition tyrosine at position 101 of the heavy chain also interacts. None of these residues are predicted to be involved in determining the main chain conformation of the antibody variable regions (Chothia and Lesk (1987) Journal of Molecular Biology 196, p901-917).

Mutagenesis of pCAT3SCFvD1.3

The oligonucleotides mutL91,92, was prepared to randomise phenylalanine at position 91 (L91) and tryptophan at position 92 (L92) of the light chain. The oligonucleotides mutL32, was prepared to randomise tyrosine at light chain position 32 (L32) and the oligonucleotides mutH101 was prepared to randomise tyrosine at position 101 of the heavy chain (H101).

mutL91,92:
5' CGT CCG AGG AGT ACT NNN NNN ATG TTG ACA GTA ATA 3'
mutL32:
5' CTG ATA CCA TGC TAA NNN ATT GTG ATT ATT CCC 3'
mutH101:
5' CCA GTA GTC AAG CCT NNN ATC TCT CTC TCT GGC 3'

(N represents a random insertion of equal amounts of A,C,G or T) in vitro mutagenesis of the phagemid vector, pCAT3scFvD1.3 (example 17) with the oligonucleotide mutL91,92 was carried out using an in vitro mutagenesis kit (Amersham). The resultant DNA was transformed by electroporation into TG1 cells using a Bio-Rad electroportor. 78,000 clones were obtained and these were scraped into 15mls of 2xTY/20% glycerol. This pool was called D1.3L91L92. Single stranded DNA was prepared by rescue with M13K07 as described in Sambrook et al, 1989 supra, and sequenced with the primer FDTSEQ1, using a Sequenase sequencing kit (United States Biochemical Corporation).

This revealed that the DNA had been successfully mutagenised as judged by the presence of bands in all four DNA sequencing tracks at the nucleotide positions encoding L91 and L92. This mutagenised single stranded DNA was subjected to a further round of mutagenesis as

above using either mutL32 or mutH101 oligonucleotides. Mutagenesis with mutL32 gave rise to 71,000 clones (pool called D1.3L32) while mutH101 gave 102,000 clones (pool called D1.3H101). These clones were scraped into 15mls of 2xTY/20% glycerol. Single stranded DNA derived from each pool was sequenced with the oligonucleotides D1.3L40 and LINKSEQ1 respectively, as described above, and shown to be correctly randomised.

D1.3L40:

5' CAG GAG CTG AGG AGA TTT TCC 3'

LINKSEQ1:

5' TCC GCC TGA ACC GCC TCC ACC 3'

Preparation of rescued phage for affinity purification

10-20µl of bacteria derived from each mutagenised pool (plate scrapes) was used to inoculate 5mls of TY/Glu/Amp. All bacterial growth was at 37°C. After 2-3 hours growth, 1ml was diluted in 5mls of prewarmed TY/Glu/Amp and infected by addition of 0.5 mls of a 200 fold concentrate of the M13K07gIII △ No.3 preparation described in example 34. After 1 hour of infection the cultures were centrifuged at 4,000 r.p.m. for 10 minutes, resuspended in 2xTY, 100µg/ml ampicillin, incubated for a further hour, transferred to 500 mls of 2xTY medium containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and grown for 16 hours. The remaining steps of phage preparation were as described in example 44. Phage were finally dissolved in 10mM Tris, 1mM EDTA pH7.4 at 1/100th the original culture volume.

Affinity purification

10mls of turkey egg lysozyme at a concentration of 10mg/ml in 0.1M NaHCO3, 0.5MNaCl pH8.3 was mixed with an equal volume of swollen Cyanogen Bromide Activated Sepharose 4B (Pharmacia), covalently linked and washed according to manufacturers instructions. Before use this matrix (TEL-Sepharose) was washed with 100 volumes of PBS followed by 10 volumes of PBSM. The TEL-Sepharose was resuspended in an equal volume of PBSM and 1ml was added to 1ml of a 50 fold concentrate of phage in PBSM and incubated on a rotating platform for 30 minutes at room temperature. The actual phage used for this step was prepared by mixing equal volumes of the independent preparations of the three randomised pools (D1.3L9192, D1.3H101 and D1.3L32). After this binding step, the suspensions were loaded onto a disposable polypropylene column (Poly-Prep columns, Bio-Rad) and washed with 200 volumes of PBS containing 0.1% Tween 20. Bound phage were eluted with 1ml of 100mM triethylamine and neutralised with 0.5ml 1M Tris (pH7.4). A dilution series was prepared from the eluate and used to infect TG1 cells and plated out on TY plates containing 100µg/ml ampicillin, 2% glucose. Plates carrying approximately 10^0 colonies were scraped into 3mls of 2xTY, 20% glycerol

and stored at -70°C. 10µl of this was used to initiate a second round culture which was rescued with M13K07gIIIΔ No.3 as described above (using a final culture volume of 100mls). Second and third round affinity column purification steps were carried out as described above for the first round.

Analysis by ELISA

40 colonies derived from the third round of column purification on TEL-Sepharose were picked into a 96 well plate and grown overnight at 37°C in 200µl of TY/Amp/Glu. Phagemid particles were rescued and prepared for ELISA as described in example 18. ELISA plates were coated overnight at 37°C with hen egg lysozyme (HEL) or turkey egg lysozyme (TEL) at a concentration of 200µg/ml in 50mM $NaHCO_3$ pH9.6 ELISAs were carried out as described in example 18.

After 15 minutes incubation in substrate, 13 clones were found to be negative (OD<0.05 on HEL and TEL). In all positives, a signal of 0.1-0.78 was scored on HEL with the exception of one where signal on HEL was 0.078 but signal on TEL (OD 0.169) brought it in to the positive group. The control phagemid preparation had a percentage ratio of signal TEL:HEL of 22%. Clones were deemed to have an unaltered binding if the ratio of TEL:HEL was less than 40%. 9 clones fell into this category. 18 samples were scored as having altered binding with a ratio of signal on TEL:HEL of between 40-200%.

A dilution series was made on 10 clones which were analysed by ELISA in 6 of these clones the profile of binding to HEL was the same as the original clone (pCAT3SCFvD1.3) while the signal with TEL was increased (see figure 50 clone B1). In the remaining 4 clones, the increased signal with TEL was accompanied by a decrease in signal on HEL (see figure 50 clone A4).

Competition with soluble antigen

All of the isolated clones retained binding to HEL to varying extents. In order to determine whether a soluble antigen could compete with the immobilised antigen, a parallel experiment was carried out, as above, but with the addition of hen egg lysozyme (1mg/ml) to TEL-Sepharose before incubating with the phage preparation. This experiment was carried through 3 rounds of column purification and 40 colonies were picked. None of these clones bound HEL or GEL demonstrating that the soluble antigen had been successful in competing out binding to the immobilised antigen.

Example 46

Modification of the Specificity of an Antibody by Replacement of the VLK Domain by a VLK Library derived from an Unimmunised Mouse

When an antibody specificity is isolated it will often be desirable to alter some of its properties particularly its affinity or specificity. This example demonstrates that the specificity of an antibody can be altered by use of a different VL domain derived form a repertoire of such domains. This method using display on phage would be applicable to improvement of existing monoclonal antibodies as well as antibody specificities derived using phage antibodies. This example shows that replacement of the VL domain of scFvD1.3 specific for Hen eggwhite lysozyme (HEL) with a library of VL domains allows selection of scFv fragments with bind also to Turkey eggwhite lysozyme (TEL). More generally this experimental approach shows that specificities of antibodies can be modified by replacement of a variable domain and gives a further example of the hierarchical approach to isolating antibody specificities.

The D1.3 heavy chain was amplified from an existing construct (pSW1-VHD1.3, Ward et al., 1989 supra) by PCR using the primers VH1BACK and VH1FOR, the light chain library was amplified from a cDNA library derived from the spleen of an unimmunised mouse, which was synthesized by using the MJKFONX primers 1,2,4,5 for the first strand as in example 14. The subsequent amplification was performed with the same forward primers and the VK2BACK primer. The PCR assembly of the D1.3 heavy chain with the light chain library was mediated by the signal chain Fv linker as described in example 14.

Cloning the assembled PCR products (scFv sequences) was done after an additional PCR step (pull-through) using a BACK primer providing an ApaLI site and forward primers which contained a Not 1 site as described in example 14. ApaL1/Not 1 digested PCR fragments were cloned into the similarly digested vector fdCAT2 as in example 11. $5x10^5$ transformations were obtained after electroporation of the ligation reaction into MC1061 cells.

Screening of the phage library for TEL binders was performed by panning. Polystyrene Falcon 2058 tubes were coated (16 hrs) with 2 ml of TEL-PBS (3 mg/ml) and blocked for 2 hrs with 4 ml MPBS (PBS containing 2% skimmed milk powder). Phage derived from the library ($5x10^{10}$ transducing unites) in 2 ml of MPBS (2%) were incubated in these tubes for 2 hrs at room temperature. The tubes were washed 3x with PBS, 1x with 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl; 1x with 50mM Tris-HCl, pH8.5, 0.5 M NaCl, 50 mM Tris-HCl, pH 9.5 M NaCl. Finally phage were eluted with 100 mM triethylamine. Eluted phages were taken to infect TG1 cells, the cells were plated on 2xTY plates containing 15 µg/ml tetracycline and grown for 16h. The colonies were scraped into 25ml of 2xTy medium and the phages were recovered by PEG

precipitation. After a second round of selection for TEL binders ELISAs were performed as described (example 2).

Analysis of 100 clones from the library before affinity selection by ELISA on plates coated with TEL showed no binders. In contrast, after two rounds of selection for TEL binding phages about 10% of the phage clones showed positive ELISA signals. ELISA signals were scored positive with values at least two fold higher than the fdCAT2 vector without insert. A more detailed analysis of binding properties of TEL binding phages is shown in figure 51.

As shown in figure 51, several clones were found which bind equally to TEL and HEL in contrast to the original D1.3 scFv, which binds almost exclusively to HEL. None of the clones bound to BSA. These findings indicate that the specificity of these scFvs was broader in comparison to D1.3, since both lysozymes (HEL and TEL) are recognized, but specificity for lysozyme was retained since other BSA was not recognized. The deduced amino acid sequences (derived by DNA sequencing) of two light chains from clones MF1 and M21, which correspond to clones 3 and 9 in figure 51 are shown in figure 52.

In the case of isolated antibodies the experimental approach as described in this study may be particularly useful if recognition of a wider range of different but closely related antigens is desired. For example, monoclonal antibodies against viral antigens viral antigens like V3 loop of HIV-1 gp120 are in most cases quite specific for one particular virus isolate because of the variability in this part of the HIV-1 env gene. The modification of such antibodies in the way described in this example may lead to antibodies which cross react with a wider range of HIV-1 isolates, and would therefore be of potentially higher therapeutic or diagnostic value.

A similar approach could be taken in which a light chain variable domain of desired properties is kept fixed and combined with a library of heavy chain variable domains. Some heavy chains, for example VHD1.3 retain binding activity as single domains. This may allow a strategy where VH domains are screened for binding activity when expressed on phage and then binding domains combined with a library of VL domains for selection of suitable light chain partners.

Example 47

Selection of a Phage Antibody Specificity by Binding to an Antigen attached to Magnetic Beads. Use of a Cleavable Reagent to allow elution of Bound Phage under Mild Conditions

When a phage antibody binds to its antigen with high affinity or avidity it may not be possible to elute the phage antibody from an affinity matrix with a molecule

related to the antigen. Alternatively, there may be no suitable specific eluting molecule that can be prepared in sufficiently high concentration. In these cases it is necessary to use an elution method which is not specific to the antigen-antibody complex. Unfortunately, some of the non-specific elution methods disrupt phage structure, for instance phage viability is reduced with time at pH12 (Rossomando, E.F. and Zinder, N.D. J. Mol. Biol. 36 387-399 1968). A method was therefore devised which allows elution of bound phage antibodies under mild conditions (reduction of a dithiol group with dithiothreitol) which do not disrupt phage structure.

Target antigen was biotinylated using a cleavable biotinylation reagent. BSA conjugated with 2-phenyl-5-oxazolone (O. Makela et al. supra) was modified using a biotinylation reagent with a cleavable dithiol group (sulphosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate from Pierce) according to the manufacturers instructions. This biotinylated antigen was bound to streptavidin coated magnetic beads and the complex used to bind phage. Streptavidin coated magnetic beads (Dynal) were precoated with antigen by mixing 650µg of biotinylated OX-BSA in 1 ml PBS, with 200µl of beads for at least 1 hour at room temperature. Free antigen was removed by washing in PBS. One fortieth of the complex (equivalent to 5µl of beads and an input of 17.5 µg of OX-BSA) was added to 0.5ml of phage in PBSM (PBS containing 2% skimmed milk powder) containing 1.9×10^{10} phage particles mixed at the ratios of pAbD1.3 directed against lysozyme (example 2) to pAbNQ11 directed against 2-phenyl-5-oxazolone (example 11) shown in Table 12.

After 1 hour of incubation with mixing at room temperature, magnetic beads were recovered using a Dynal MPC-E magnetic desperation device. They were then washed in PBS containing 0.5% Tween 20, (3x10 minutes, 2x1 hour, 2x 10 minutes) and phage eluted by 5 minutes incubation in 50µl PBS containing 10mM dithiothreitol. The eluate was used to infect TG1 cells and the resulting colonies probed with the oligo NQ11CDR3
(5' AAACCAGGCCCCGTAATCATAGCC 3')
derived from CDR3 of the NQ11 antibody (This hybridises to pAbNO11 but not pAb D1.3).

A 670 fold enrichment of pAbNQ11 (table 12) was achieved form a background of pAbD1.3 in a single round of purification using the equivalent of 17.5µg of biotinylated OX-BSA.

This elution procedure is just one example of an elution procedure under mild conditions. A particularly advantageous method would be to introduce a nucleotide sequence encoding amino acids constituting a recognition site for cleavage by a highly specific protease between the foreign gene inserted, in this instance a gene for an

antibody fragment, and the sequence of the remainder of gene III. Examples of such highly specific proteases are Factor X and thrombin. After binding of the phage to an affinity matrix and elution to remove non-specific binding phage and weak binding phage, the strongly bound phage would be removed by washing the column with protease under conditions suitable for digestion at the cleavage site. This would cleave the antibody fragment from the phage particle eluting the phage. These phage would be expected to be infective since the only protease site should be the one specifically introduced. Strongly binding phage could then be recovered by infecting e.g. E.coli TG1 cells.

Example 48

Use of Cell Selection to provide an Enriched Pool of Antigen Specific Antibody Genes, Application to reducing the Complexity of Repertoires of Antibody Fragment displayed on the Surface of Bacteriophage

There are approximately 10^{14} different combinations of heavy and light chains derived from the spleen of an immunised mouse. If the random combinatorial approach is used to clone heavy and light chain fragments into a single vector to display scFv, Fv or Fab fragments on phage, it is not a practical proposition to display all 10^{14} combinations. One approach, described in this example, to reducing the complexity is to clone genes only from antigen selected cells. (An alternative approach, which copes with the complexity is the dual combinatorial library described in example 26).

The immune system uses the binding of antigen by surface immunoglobulin to select the population of cells that respond to produce specific antibody. This approach of selecting antigen binding cells has been investigated to reduce the number of combinatorial possibilities and so increase the chance of recovering the original combination of heavy and light chains.

The immunological response to the hapten 4-hydroxy-3-nitrophenylacetic acid (NP) has been extensively studied. Since the primary immune response to NP uses only a single light chain the applicants were able to examine the use of the combinatorial method using a fixed light chain and a library of heavy chains to examine the frequencies genes that code for antibodies binding to NIP (4-hydroxy-3-iodo-5-nitrophenylacetic acid). The applicants have thus used this system to investigate the merits of selecting cell populations prior to making combinatorial libraries for display on phage.

Methods

2.1 Hapten conjugates

Chick gamma globulin (CGG, Sigma, Poole, UK) and Bovine serum albumen (BSA, Boehringer, Mannheim, Germany) were conjugated with NP-O-succinimide or NIP-caproate-O-

succinimide (Cambridge Research Biochemicals, Northwich, UK) based on the method described by Brownstone (Brownstone, A., Mitchison, N.A. and Pitt-Rivers, R., Immunology 1966. 10: 465-492). The activated compounds were dissolved in dimethylformamide and added to proteins in 0.2 M sodium hydrogen carbonate. They were mixed with constant agitation for 16 hours at 4°C and then dialysed against several changes of 0.2 M sodium hydrogen carbonate. They were finally dialysed into phosphate buffered saline (PBS). The conjugates made were $NP_{12}CGG$, $NIP_{10}BSA$. The $NIP_{10}BSA$ derivative was subsequently biotinylated using a biotinylation kit purchased from Amersham (Amersham International, Amersham, UK).

2.2 Animals and immunisation

Mice of the strain C57BL/6 were immunised by intraperitoneal injection of 100µg NP-CGG in Complete Freunds Adjuvant at 10 weeks of age.

2.3 Spleen preparation

Seven days after immunization cells from the spleen were prepared as described by Galfre and Milstein (Galfre, G. and Milstein, C. Methods Enzymol. 1981. 73:3-46). Red cells were lysed with ammonium chloride (Boyle, W. Transplantation 1968.6:71) and when cell selection was performed dead cells were removed by the method described by von Boehmer and Shortman (von Boehmer, H. and Shortman, K, J. Immunol, Methods 1973:1:273). The cells were suspended in phosphage buffered saline (PBS), 1% Bovine serum albumen, 0.01% sodium azide; throughout all cell selection procedures the cells were kept at 4°C in this medium.

2.4 Cell Solution

Biotinylated NIP-BSA was coupled to streptavidin coupled magnetic beads (Dynabeads M280 Streptavidin, Dynal, Oslo, Norway) by incubating 10^8 beads with 100µg of biotinylated protein for 1 hour, with occasional agitation, and then washing five times to remove unbound antigen. The coupled beads were stored at 4°C in medium until required. For selection of antigen binding cells the cells ($2\text{-}4\times10^7$/ml) were first incubated for 30 minutes with uncoupled beads, at a bead: cell ratio of 1:1, to examine the degree of non-specific binding. The beads were then separated by placing the tube in a magnetic device (MPC-E Dynal) for 3-5 minutes. The unbound cells were removed and then incubated with NIP-BSA coupled magnetic beads, at a bead:cell ratio of 0.1:1, for 60 minutes, with occasional agitation. The beads and rosetted cells were separated as described above. The beads were then resuspended in 1 ml of medium and the separation repeated; this process was repeated 5-7 times until no unbound cells could be detected when counted on a haemocytometer.

For the depletion of surface immunoglobulin positive

cells the cells were incubated with 20µg biotinylated goat anti-mouse polyvalent immunoglobulin (Sigma, Poole, UK). The cells were then washed twice with medium and added to streptavidivin coupled magnetic beads at a bead to cell ratio of 30:1. After 30 minutes incubation the beads and rosetted cells were separated by applying the magnetic device three times - taking the supernatant each time.

2.4 DNA/cDNA preparation, PCR amplification and cloning

DNA was prepared by a simple proteinase-K digest method that was particularly convenient for small numbers of cells (PCR Protocols: A Guide to Methods and Applications. Ed Innis M.A., Gelfand D. H., Sninsky J.J. and White T. J. Academic Press). RNA preparation and subsequent cDNA synthesis was performed as described by Gherardi et al (Gherardi E., Pannell R. and Milstein C. J. Immunol. Methods, 1990. 126:61-68). PCR and cloning of the heavy chain libraries was performed using the primers and conditions described by Ward et al (Ward, E.S., Güssow, D., Griffiths, A.D., Jones, P.T. and Winter, G., Nature, 1989. 341: 544-546); 40 cycles of PCR amplification were performed. The VH and Fv expression vectors used were adapted from those previously described by Ward et al. They were both subcloned into pUC119 (Veira and Messing see later) and the Fv expression vector was modified to include a germline lambda-1 light chain (obtained as a gift from T. Simon (originally cloned by Siegfried Weiss, Basel Institute of Immunology)). THe vector is shown in Figure 53.

2.5 Expression and ELISA

For screening single colonies were picked into individual wells of microtitre plates (Bibby) in 200µl 2 x TY/Ampicillin 100µg/ml/0.1% glucose and then incubated at 37°C for 5-6 hours with agitation, Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, Poole, UK) was then added to a final concentration of 1 mM and the incubation continued for a further 16 hours at 30°C before harvesting the supernatants. The wells of Falcon ELISA plates (Becton Dickenson, N.J., USA) were coated overnight at room temperature with NIP_{10}-BSA (40µg/ml in PBS) and then blocked with 2% skimmed milk powder in PBS for 2 hours at room temperature. The bacterial supernatants were added and incubated at room temperature for 1 hour and then the plates were washed three times with PBS. Peroxidase conjugated-Goat anti-mouse lambda-chain (Southern Biotechnology, Birmingham, USA) was added and again incubated for 1 hour at room temperature before washing six times with PBS and then developing with 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, Poole, UK) as the peroxidase substrate. The optical density at 405nm was measured using a Thermomax

microplate reader (Molecular Devices, Menlo Park, USA) after 30 minutes. Western blotting using the C-terminal myc tag as described in example 27.

3.1 Comparison of RNA/DNA and antigen selected cells

The results of antigen selection are shown in Table 13. Less than 1% of cells bind to NIP-BSA coated beads and the non-specific binding is very low. Assessment of the proportion of expressed genes from each VH library using western blotting showed that full length VH domains were expressed in 95% (19/20) of all clones when RNA was used as the starting material but only 60% (12/20) of clones when DNA (either selected cells or from total spleen) was used as the starting material. This difference probably results from the fact that many re-arranged pseudogenes could be amplified with our primers and it appears that there must be some degree of selection, at the level of transcription, for functional genes.

A variable number of clones from each type of library were screened for the production of Fv fragments that bound to NIP. Initial screening ELISAs were performed and positives taken to include those with an optical density of at least twice the background. The initial positives were retransformed and the binding checked in duplicate; it was confirmed that the binding was specific to NIP and not to BSA. The frequency of confirmed positive NIP binding clones for each starting material are shown in Table 14. Using DNA as the starting material for the PCR amplification is approximately equivalent to sampling the cells present as there is only one functional re-arranged heavy chain gene and at most one re-arranged pseudogene per B-cell. Amplifying from the RNA of an animal of course biases the repertoire to the reacting B-cells and in a recently immunised animal this would be expected to give some bias towards the immunogen. The data in Table 14 clearly shows how powerful this selection is with the number of antigen specific genes being enriched at least 96 fold when RNA made one week after primary immunisation is used as the starting material. The data also show that selection for antigen binding cells also provides an alternative powerful method of selection for the required genetic starting material.

3.2 Comparison of Total Spleen/surface immunoglobulin depleted Spleen

To examine the cellular basis of the selection achieved by using RNA as the starting material we depleted the spleen of surface immunoglobulin positive cells using biotinylated anti-polyvalent immunoglobulin and streptavidin conjugated magnetic beads. Prior FACS analysis had demonstrated that this method removed over 96% of surface immunoglobulin positive cells. RNA was

prepared from both surface immunoglobulin depleted and non-depleted factions of a spleen and VH libraries made from each. The ELISA results (Table 14) show that the number of positives is certainly not decreased by this depletion suggesting that the major portion of the selective effect of using RNA may come from surface immunoglobulin negative G-cells (probably plasma cells).

Conclusions

The applicants have demonstrated the importance of the amplification of specific RNA produced by immunisation to enable binding activity to be obtained with any reasonable frequency from a combinatorial library. The applicants have also demonstrated an alternative strategy which mimics that of the immune system itself. Using a simple method of selecting for antigen binding cells gave comparable enrichment and has the added advantage of using a broader range of genes. At first sight the random combinatorial approach would appear unlikely to produce the original combination of heavy and light chain because of the vast diversity of the immunoglobulin genes. The applicants show here, however, that following immunisation, with a good antigen, 10% of the VH genes from total splenic RNA isolated come from antigen specific cells so the effective size of the repertoire is greatly reduced. This together with the fact that promiscuity of the heavy and light chains occurs (examples 21 and 22) accounts for the fact that combinatorial system does produce antigen binding clones with reasonable frequency. The data also suggests that the bulk of the antigen specific RNA comes from surface immunoglobulin negative cells which are most likely plasma cells.

The data also show that this simple method of antigen selection may be useful in reducing the complexity of the combinatorial library. In this case an enrichment of antigen specific genes of at least 56 fold has been achieved which in the normal case where heavy and light chains are unknown would result in a reduction of the complexity of the combinatorial library by a factor of over 3000. A further advantage of using antigen selected cells (and amplifying from DNA to reduce any bias due to the state of the cell) is that this results in a broader range of antibody genes amplified. It may be that a simple cell selection such as that the applicants have described here in combination with phage selection would be ideal. From this example it can be seen that by combining cell and phage selection methods one could reasonably expect to screen all the combinations of heavy and light chain (approximately $4x10^{10}$) and would thus be able to screen all binding combinations although this would not, at present, be possible from whole spleen (approximately $4x10^{14}$ combinations, assuming 50% B-cells).

Table 1. Enrichment of pAb (D1.3) from vector population

INPUT RATIO[a]	OUTPUT RATIO		ENRICHMENT[d]
	oligo[b]	ELISA[c]	
pAb:fd-CAT1	pAb:total phage	pAb:total phage	
Single Round			
$1:4x10^3$	43/124		$1.3x10^3$
$1:4x10^4$	2/82		$1.0x10^3$
Two Rounds			
$1:4x10^4$	197/372		$2.1x10^4$
$1:4x10^5$	90/356	3/24	$1.0x10^5$
$1:4x10^6$	27/183	5/26	$5.9x10^5$
$1:4x10^7$	13/278		$1.8x10^6$

Footnotes: [a]Approximately 10^{12} phage with the stated ratio of pAb (D1.3) : FDTPs/Bs were applied to 1 ml lysozyme-sepharose columns, washed and eluted. [b]TG1 cells were infected with the eluted specific binding phage and plated onto TY-tet plates. After overnight incubation at 30-37°C, the plates were analysed by hybridisation to the ^{32}p, labelled oligonucleotide VH1FOR (Ward et al op cit) which is specific to pAb D1.3. [c] Single colonies from overnight plates were grown, phage purified, and tested for lysozyme binding. [d]Enrichment was calculated from the oligonucleotide probing data.

Table 2 Enrichment of pAb (D1.3) from mixed pAb population

Input Ratio1 (pAbD1.3:pAbNQ11)	Output Ratio[2] (pAb D1.3:Total phage)	Enrichment
Single Round		
1 : 2.5 x 10^4	18/460	0.98 x 10^3
1 : 2.5 x 10^5	3/770	0.97 x 10^3
1 : 2.5 x 10^6	0/112	-
pAb NQ11 only	0/460	-
Second Round		
1 : 2.5 x 10^4	119/170	1.75 x 10^4
1 : 2.5 x 10^5	101/130	1.95 x 10^5
1 : 2.5 x 10^6	102/204	1.26 x 10^6
1 : 2.5 x 10^7	0/274	-
1 : 2.5 x 10^8	0/209	-
pAb NQ11 only	0/170	-

Notes

1. 10^{10} phage applied to a lysozyme column as in table 1.
2. Plating of cells and probing with oligonucleotide as in table 1, except the oligonucleotide was D1.3CDR3A.

Table 3: Enzyme activity of phage-enzyme

Input	ng of enzyme or No. of phage	Rate (OD/hr)	No. of molecules of Enzyme equivalent ($x10^{-11}$)
Pure Enzyme	335	34	24.5
Pure Enzyme	177.5	17.4	12.25
Pure Enzyme	88.7	8.7	6.125
Pure Enzyme	44.4	4.12	3.06
Pure Enzyme	22.2	1.8	1.5
Pure Enzyme	11.1	0.86	0.76
No Enzyme	0	0.005	0
fd-phoAla166/TG1	$1.83x10^{11}$	5.82	4.2
fd-CAT2/TG1	$1.0x10^{12}$	0.155	0.112
fd-phoAla166/KS272	$7.1x10^{10}$	10.32	7.35
fd-CAT2/KS272	$8.2x10^{12}$	0.038	0.027

Table 4. Affinity selection of hapten-binding phage.

		Clones binding to phOx[†]			
		Pre-column	After first round	After second round	After third round
A	**Random Combinatorial Libraries**				
	phOx-immunised mice	0/568 (0%)	48/376 (13%)	175/188 (93%)	-
	Unimmunised mice	-	-	0/388 (0%)	-
B	**Hierarchical Libraries**				
	VH-B / Vκ-*rep* library	6/190 (3%)	348/380 (92%)	-	-
	VH-*rep* / Vκ-d library	0/190 (0%)	23/380 (7%)	-	-
C	**Fractionation of VH-B/Vκ-d and VH-B/Vκ-b phage[†]**				
	Mixture of clones	88/1896 (4.6%) [44/1740 (2.5%)[*]]	55/95 (57.9%)	1152/1156 (99.7%)	1296/1299 (99.8%)

[†] In panel C, numbers refer to VH-B/Vκ-d colonies.

[*] Numbers after three reinfections and cycles of growth. This control, omitting the column steps, confirms that a spurious growth or infectivity advantage was not responsible for the enrichment for clone VH-B/Vκ-d.

Table 5

	Phage/Phagemid†	Helper phage	Binding to phOx*	Chain(s) displayed#	Chain as gene III fusion#	Soluble chain(s)#
A	fd CAT2		non binding	none		
	fd CAT2-I		binding	scFv	scFv	
	fd CAT2-II		binding	Fab	light chain	heavy chain
	pHEN1	VCSM13	non binding	none		
	pHEN1-I	VCSM13	binding	scFv	scFv	
	pHEN1-II	VCSM13	binding	Fab	light chain	heavy chain
B	pHEN1 I (HB2151)		binding			scFv§
	pHEN1 II (HB2151)		binding			Fab§
C	fd CAT2-III		non binding	heavy chain	heavy chain	
	fd CAT2-IV		non binding	light chain	light chain	
	pHEN1-III (HB2151)	VCSM13	non binding	none		heavy chain
	pHEN1-III (HB2151)	fd-tet-DOG1-IV	binding	Fab	light chain	heavy chain
	pHEN1-IV (HB2151)	VCSM13	non binding	none		light chain
	pHEN1 IV (HB2151)	fd tet-DOG1 III	binding	Fab	heavy chain	light chain

Overview of phOx-BSA ELISA results of phage and phagemid constructions.
* Phage were considered to be 'binding' if OD_{405} of sample was at least 10 fold greater than background in ELISA; † *E.coli* TG1 was used for the growth of the phage unless the use of *E.coli* HB2151 is specifically indicated; # Information deduced from genetic structure and in accordance with binding data; § Result confirmed experimentally by Western blot (for Fab, see Figure 29.

	Soluble enzyme (Data from Chaidaroglu et al 1988)		Phage enzyme (Data from this study)	
	phoArg166	phoAla166	phoArg166	phoAla166
K_m (μM)	12.7	1620	73	1070
Relative K_m	1	127	1	14.6
Relative k_{cat}	1	0.397	1	0.360
Relative k_{cat}/K_m	1	0.0032	1	0.024

Table 6. Kinetic parameters of soluble and phage-bound alkaline phosphatase. Relative values of k_{cat} and K_m for the soluble enzyme and for the phage enzyme were derived by comparing with the values for wild type enzyme (phoArg166) and the phage-wild type enzyme (fdphoArg166).

Enzyme Activity of Phage Samples

SAMPLE (Construct:host)	INPUT PHAGE PARTICLE (pmol)	RATE (pmol substrate converted/min)	SPECIFIC ACTIVITY (mol substrate converted/mol phage/min)
fdphoArg166 :TG1	2.3	8695	3700
fdphoAla166 :TG1	5.6	2111	380
fdphoAla166 :KS272	1.8	2505	1400
fdCAT2: TG1	3.3	<1	<0.3
fdCAT2: KS272	5.6	70	12

Table 7

Table 8. Affinity chromatography of phage-enzymes

SAMPLE	INFECTIVITY (Percentage of phage particles which are infectious)	INPUT PHAGE PARTICLE ($x10^9$)	OUTPUT PHAGE PARTICLE ($x10^9$)
fdphoArg166	0.37%	5160	30
fdphoAla166	0.26%	3040	90
fdCAT2	4.75%	4000	2

Nucleotide mutation (base position)	Amino acid mutation	Number
308	Ala->Val (VH FR3)	3
703	Tyr->Asp (VL CDR3)	1
706	Ser-> Gly (VL CDR3)	1
724	Gly-> Ser (VL FR4)	21
725	Gly-> Asp (VL FR4)	3
734	Thr-> Ile (VL FR4)	1

Table 9 Mutations in scFvB18 selected by display on phage following growth in mutator strains

Table 10(i) Oligonucleotide primers used for PCR of human immunoglobulin genes

Oligo Name	Sequence
Human VH Back Primers	
HuVH1aBACK	5'-CAG GTG CAG CTG GTG CAG TCT GG-3'
HuVH2aBACK	5'-CAG GTC AAC TTA AGG GAG TCT GG-3'
HuVH3aBACK	5'-GAG GTG CAG CTG GTG GAG TCT GG-3'
HuVH4aBACK	5'-CAG GTG CAG CTG CAG GAG TCG GG-3'
HuVH5aBACK	5'-GAG GTG CAG CTG TTG CAG TCT GC-3'
HuVH6aBACK	5'-CAG GTA CAG CTG CAG CAG TCA GG-3'
HuVH1aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG GTG CAG TCT GG-3'
HuVH2aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTC AAC TTA AGG GAG TCT GG-3'
HuVH3aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG TCT GG-3'
HuVH4aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GAG TCG GG-3'
HuVH5aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG TTG CAG TCT GC-3'
HuVH6aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTA CAG CTG CAG CAG TCA GG-3'
Human JH Forward Primers	
HuJH1-2FOR	5'-TGA GGA GAC GGT GAC CAG GGT GCC-3'
HuJH3FOR	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
HuJH4-5FOR	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
HuJH6FOR	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'
Human Heavy Chain Constant Region Primers	

Table 10 Cont.)

HuIgG1-4CH1FOR	5'-GTC CAC CTT GGT GTT GCT GGG CTT-3'
HuIgMFOR	5'-TGG AAG AGG CAC GTT CTT TTC TTT-3'

Human Vκ Back Primers

HuVκ1aBACK	5'-GAC ATC CAG ATG ACC CAG TCT CC-3'
HuVκ2aBACK	5'-GAT GTT GTG ATG ACT CAG TCT CC-3'
HuVκ3aBACK	5'-GAA ATT GTG TTG ACG CAG TCT CC-3'
HuVκ4aBACK	5'-GAC ATC GTG ATG ACC CAG TCT CC-3'
HuVκ5aBACK	5'-GAA ACG ACA CTC ACG CAG TCT CC-3'
HuVκ6aBACK	5'-GAA ATT GTG CTG ACT CAG TCT CC-3'

Human Jκ Forward Primers

HuJκ1FOR	5'-ACG TTT GAT TTC CAC CTT GGT CCC-3'
HuJκ2FOR	5'-ACG TTT GAT CTC CAG CTT GGT CCC-3'
HuJκ3FOR	5'-ACG TTT GAT ATC CAC TTT GGT CCC-3'
HuJκ4FOR	5'-ACG TTT GAT CTC CAC CTT GGT CCC-3'
HuJκ5FOR	5'-ACG TTT AAT CTC CAG TCG TGT CCC-3'

HuJκ1BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC-3'
HuJκ2BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAG CTT GGT CCC-3'
HuJκ3BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC TTT GGT CCC-3'
HuJκ4BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAC CTT GGT CCC-3'
HuJκ5BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC-3'

Human κ Constant Region Primers

Table 10 (iii)

HuCκFOR	5'-AGA CTC TCC CCT GTT GAA GCT CTT-3'
HuCκFORNot1	5'-GAG TCA TTC TCG ACT TGC GGC CGC TTA TTA AGA CTC TCC CCT GTT GAA GCT CTT-3'
HuCκFORNot2	5'-GAG TCA TTC TCG ACT TGC GGC CGC AGA CTC TCC CCT GTT GAA GCT CTT-3'

Human λ Back primers

Huλ1BACK	5'-CAG TCT GTG TTG ACG CAG CCG CC-3'
Huλ2BACK	5'-CAG TCT GCC CTG ACT CAG CCT GC-3'
Huλ3aBACK	5'-TCC TAT GTG CTG ACT CAG CCA CC-3'
Huλ3bBACK	5'-TCT TCT GAG CTG ACT CAG GAC CC-3'
Huλ4BACK	5'-CAC GTT ATA CTG ACT CAA CCG CC-3'
Huλ5BACK	5'-CAG GCT GTG CTC ACT CAG CCG TC-3'
Huλ6BACK	5'-AAT TTT ATG CTG ACT CAG CCC CA-3'

Human λ Forward Primers

Hu Jλ1FOR	5'-ACC TAG GAC GGT GAC CTT GGT CCC-3'
Hu Jλ2-3FOR	5'-ACC TAG GAC GGT CAG CTT GGT CCC-3'
Hu Jλ4-5FOR	5'-ACC TAA AAC GGT GAG CTG GGT CCC-3'

Hu Jλ1FORNOT	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT CCC-3'
Hu Jλ2-3FORNOT	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT CAG CTT GGT CCC-3'
Hu Jλ4-5FORNOT	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACY TAA AAC GGT GAG CTG GGT CCC-3'

Human λ Constant Region Primers

Table 10

HuCλFOR	5'-TGA AGA TTC TGT AGG GGC CAC TGT CTT-3'
HuCλFORNot1	5'-GAG TCA TTC TCG ACT TGC GGC CGC TTA TTA TGA AGA TTC TGT AGG GGC CAC TGT CTT-3'
HuCλFORNot2	5'-GAG TCA TTC TCG ACT TGC GGC CGC TGC AGA TTC TGT AGG GGC TGT CTT-3'

Linker oligos

Reverse JH for scFv linker

RHuJH1-2	5'-GCA CCC TGG TCA CCG TCT CCT CAG GTG G-3'
RHuJH3	5'-GGA CAA TGG TCA CCG TCT CTT CAG GTG G-3'
RHuJH4-5	5'-GAA CCC TGG TCA CCG TCT CCT CAG GTG G-3'
RHuJH6	5'-GGA CCA CGG TCA CCG TCT CCT CAG GTG C-3'

Reverse IgG1-4CHH primer for Fab linker

RHuIgG1-4CHHFOR	5'-AAG CCC AGC AAC ACC AAG GTG GAC-3'

Reverse Vκ for scFv linker

RHuVκ1aBACKFv	5'-GGA GAC TGG GTC ATC TGG ATG TCC GAT CCG CC-3'
RHuVκ2aBACKFv	5'-GGA GAC TGA GTC ATC ACA ACA TCC GAT CCG CC-3'
RHuVκ3aBACKFv	5'-GGA GAC TGC GTC AAC ACA ATT TCC GAT CCG CC-3'
RHuVκ4aBACKFv	5'-GGA GAC TGG GTC ATC ACG ATG TCC GAT CCG CC-3'
RHuVκ5aBACKFv	5'-GGA GAC TGC GTG AGT GTC GTT TCC GAT CCG CC-3'
RHuVκ6aBACKFv	5'-GGA GAC TGA GTC AGC ACA ATT TCC GAT CCG CC-3'

Reverse Vκ for Fab linker

Table 10

RIIuVκ1aBACKFab 5'-GGA GAC TGG GTC ATC TGG ATG TCG GCC ATC GCT GG-3'
RIIuVκ2aBACKFab 5'-GGA GAC TGC GTC ATC ACA ACA TCG GCC ATC GCT GG-3'
RIIuVκ3aBACKFab 5'-GGA GAC TGC GTC AAC ACA ATT TCG GCC ATC GCT GG-3'
RIIuVκ4aBACKFab 5'-GGA GAC TGG GTC ATC ACG ATG TCG GCC ATC GCT GG-3'
RIIuVκ5aBACKFab 5'-GGA GAC TGC GTG AGT GTC GTT TCG GCC ATC GCT GG-3'
RIIuVκ6aBACKFab 5'-GGA GAC TGC GTC AGC ACA ATT TCG GCC ATC GCT GG-3'

Reverse Vλ for svFv linker

RIIuVλBACK1Fv 5'-GGC GGC TGC GTC AAC ACA GAC TGC GAT CCG CCA CCG CCA GAG-3'
RIIuVλBACK2Fv 5'-GCA GGC TGA GTC AGA GCA GAC TGC GAT CCG CCA CCG CCA GAG-3'
RIIuVλBACK3aFv 5'-GGT GGC TGA GTC AGC ACA TAG GAC GAT CCG CCA CCG CCA GAG-3'
RIIuVλBACK3bFv 5'-GGG TCC TGA GTC AGC TCA GAA GAC GAT CCG CCA CCG CCA GAG-3'
RIIuVλBACK4Fv 5'-GGC GGT TGA GTC AGT ATA ACG TGC GAT CCG CCA CCG CCA GAG-3'
RIIuVλBACK5Fv 5'-GAC GGC TGA GTC AGC ACA GAC TGC GAT CCG CCA CCG CCA GAG-3'
RIIuVλBACK6Fv 5'-TGG GGC TGA GTC AGC ATA AAA TTC GAT CCG CCA CCG CCA GAG-3'

Reverse Vλ for Fab linker

RIIuVλBACK1Fab 5'-GGC GGC TGC GTC AAC ACA GAC TGG GCC ATC GCT GGT TGG GCA-3'
RIIuVλBACK2Fab 5'-GCA GGC TGA GTC AGA GCA GAC TGG GCC ATC GCT GGT TGG GCA-3'
RIIuVλBACK3aFab 5'-GGT GGC TGA GTC AGC ACA TAG GAG GCC ATC GCT GGT TGG GCA-3'
RIIuVλBACK3bFab 5'-GGG TCC TGA GTC AGC TCA GAA GAG GCC ATC GCT GGT TGG GCA-3'
RIIuVλBACK4Fab 5'-GGC GGT TGA GTC AGT ATA ACG TGG GCC ATC GCT GGT TGG GCA-3'
RIIuVλBACK5Fab 5'-GAC GGC TGA GTC AGC ACA GAC TGG GCC ATC GCT GGT TGG GCA-3'
RIIuVλBACK6Fab 5'-TGG GGC TGA GTC AGC ATA AAA TTG GCC ATC GCT GGT TGG GCA-3'

Table 11. Deduced protein sequences of heavy and light chains selected from unimmunized library

Oxazolone binder

HEAVY CHAIN										
VH15.4	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	SYGIS	WVRQAPGQGLEWMG	WISAYNGNTKYAQKLQG	RVTMTTDTSTSTAYMELRSLRSDDTAVYYCVR	LLPKRTATLH	YYIDVWGKGT			
LIGHT CHAIN										
VL15.4		NNYVS	WYQHLPGTAPNLLIY	DNNKRPS	GIPDRFSGSKSGTSATLGITGLQTGDEADYYC	GIWDGR				

BSA Binders

HEAVY CHAINS										
VH3.5	QVQLVQSGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	TGYSSGWGY	FDYWGQGT			
LIGHT CHAINS										
VL3.5	SSELTQDPAVSVALGQTVRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNH	VVFGG			

Lysozyme binders:

HEAVY CHAINS										
VH10.1	SLTCSVSGDSIS	SGGYS	WIRQPSGKGLEWIG	SVHHSGPTYYNPSLKS	RVTMSVDTSKNQFSLKLKSVTAADTAMYFCAR	EGGSTWRSLYKH	YYMDVWGK			
VH14.1	QVQLQESGPGLVKPSETLSLVCTVSGGSLS	FSYWG	WIRQPPGKGLEWIG	YISHRGTDYNSSLQS	RVTISADTSKNQFSLKLSSVTAADTAVYYCAR	SFSNSFFFGY	WGQGT			
VH13.1	QVQLVQSGAEVKKPGQSLMISCQGSGYSFS	NYWIG	WVRQMPGKGLEWMG	IIYPGDSDTRYSPSFQG	QVTISADKSISTAYLHWSSLKASDTALYYCAR	LVGGTPAY	WGQGT			
VH16.1	QVQLVQSGAEVKKPGQSLRISCKGAGYSFS	TYWIG	WVRQMPGKGLEWMG	IIYPDDSDTRYSPSFEG	QVTISVDKSITTAYLHWSSLKA					
LIGHT CHAINS										
VK10.1	EIVLTQSPSSLSASVGDRVTITC	RASQSISNYLN	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTINSLQPEDFATYYC	QQTNSFP	LTFGGG			
VL14.1	SSELTQDPAVSVAFGQTVRITC	QGDSLRSSYAS	WYQQKPGQAPLLVIY	GENSRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSRGTHL	EVFGG			
VL13.1	HVILTQPASVSGSPGQSITISC	TGSSRDVGGYNYVS	WYQHHPGKAPKLLIS	EVTNRPS	GVSNRFSGSKSGNTASLTISGLQAEDEADYFC	ASYTSSRT	YVFGG			
VL16.1	QSALTQPASVSGSPGQSITISC	SGSSSDIGRYDYVS	WYQHYPDKAPKLLIY	EVKHRPS	GISHRFSASKSGNTASLTISELQPGDEADYYC	ASYT				

Table 12

Enrichment of pAbNQ11 from pAbD1.3 background by affinity selection using Ox-BSA biotinylated with a cleavable reagent and binding to streptavidin magnetic beads

Input Ratio[1] (pAbD1.3:pAbNQ11)	Output Ratio[2] (pAb NQ11: Total phage)	Enrichment
2235:1	61/197	690
22350:1	5/202	544

1. 1.9×10^{10} phage in 0.5ml mixed for 1hour with 5µl streptavidin-magnetic beads precoated with antigen (OX-BSA).
2. Colonies probed with the oligonucleotide NQ11CDR3

Table 13 : Results of antigenic cell selection

	Number of Cells	% of total cells
Total spleen cells	$4x10^7$	-
Cells bound to uncoated beads	$0.8x10^4$	0.02
Cells bound to NIP-BSA coated beads	$22x10^4$	0.55

Table 14: Results of Fv NIP binding ELISAs from selected cell populations:

	Positives	*Degree of Enrichment
Cell Population		
DNA from total spleen	0/940	-
RNA from total Spleen	29/282	> 96
DNA from antigen binding cells	17/282	>56
Surface Ig Selection		
RNA fromSurface Ig negative fraction	8/94	-
RNA from total Spleen	4/94	-

* Degree of enrichment compared to total DNA.

CLAIMS

1. A method of producing a multimeric member of a specific binding pair (sbp), which method comprises: expressing in a recombinant host organism a first polypeptide chain of said sbp member or a genetically diverse population of that type of sbp member fused to a component of a secreted replicable genetic display package (rgdp) which thereby displays said polypeptide at the surface of the package, and expressing in a recombinant host organism a second polypeptide chain of said multimer and causing or allowing the polypeptide chains to come together to form said multimer as part of said rgdp, at least one of said polypeptide chains being expressed from nucleic acid that is capable of being packaged using said component therefor, whereby the genetic material of each said rgdp encodes a said polypeptide chain.

2. A method according to claim 1 wherein both said chains are expressed in the same host organism.

3. A method according to claim 2 wherein said first and second chains of said multimer are expressed as separate chains from a single vector containing their respective nucleic acid.

4. A method according to any one of claims 1, 2 and 3 wherein at least one of said polypeptide chains is expressed from a phage vector.

5. A method according to any one of claims 1 to 4 wherein at least one of said polypeptide chains is expressed from a phagemid vector, the method including using a helper phage, or a plasmid expressing complementing phage genes, to help package said phagemid genome, and said component of the rgdp is a capsid protein therefor.

6. A method according to claim 5 wherein said capsid protein is absent, defective or conditionally defective in the helper phage.

7. A method according to any one of the preceding claims which comprises introducing a vector capable of expressing said first polypeptide chain into a host organism which expresses said second polypeptide chain in free form, or introducing a vector capable of expressing said second polypeptide in free form into a host organism which expresses said first polypeptide chain.

8. A method according to any one the preceding claims wherein each said polypeptide chain is expressed from nucleic acid which is capable of being packaged as a rgdp using said component fusion product, whereby encoding nucleic acid for both said polypeptide chains are packaged in respective rgdps.

9. A method according to any one of the preceding claims wherein the nucleic acid encoding at least one of said first and second polypeptide chains is obtained from a library of nucleic acid including nucleic acid encoding said chain or a population of variants of said chain.

10. A method according to claim 9 wherein both the first and second polypeptide chains are obtained from respective said libraries of nucleic acid.

11. A method of producing a member of a specific binding pair (sbp) from a nucleic acid library including nucleic acid encoding said sbp member or a genetically diverse population of that type of sbp member, which method comprises:
expressing in recombinant host cells polypeptides encoded by said library nucleic acid fused to a component of a secreted replicable genetic display package (rgdp) or in free form for association with a polypeptide component of said sbp member which is expressed as a fusion to said rgdp component, so that the rgdp displays said sbp member in functional form at the surface of the package, said library nucleic acid being contained within the host cells in a form that is capable of being packaged using said rgdp component, whereby the genetic material of an rgdp displaying an sbp member contains nucleic acid encoding said sbp member or a polypeptide component thereof.

12. A method of producing a member of a specific binding pair (sbp), which method comprises:
expressing in recombinant host cells nucleic acid encoding said sbp member or a genetically diverse population of that type of sbp member, wherein the or each said sbp member or a polypeptide component thereof is expressed as a fusion with a component of a secreted replicable genetic display package (rgdp) which displays said sbp member at the surface of the package, nucleic acid encoding said sbp member or a polypeptide component thereof being contained within the host cell in a form that is capable of being packaged using said rgdp component, whereby the genetic material of the rgdp displaying said sbp member encodes said sbp member or a polypeptide component thereof, said host organism being a mutator strain which introduces genetic diversity into the sbp member to produce said mixed population.

13. A method of producing a member of a specific binding pair (sbp), which method comprises:
expressing in recombinant host cells nucleic acid encoding said sbp member or a genetically diverse population of that type of sbp member, wherein the or each said sbp member or a polypeptide component thereof is expressed as a fusion with a component of a secreted replicable genetic display package (rgdp) which displays said sbp member in functional form at the surface of the package, nucleic acid encoding said sbp member or a polypeptide component thereof being

contained within the host cell in a form that is capable of being packaged using said rgdp component, whereby the genetic material of the rgdp displaying an sbp member encodes said sbp member or a polypeptide component thereof, said fusions being with bacteriophage capsid protein and the rgdps being formed with said fusions in the absence of said capsid protein expressed in wild-type form.

14. A method of producing a member of a specific binding pair (sbp) which method comprises:
expressing in recombinant host cells nucleic acid encoding said sbp member or a genetically diverse population of that type of sbp member, wherein the or each said sbp member or a polypeptide component thereof is expressed as a fusion with a component of a secreted replicable genetic display package (rgdp) which displays said sbp member at the surface of the package, nucleic acid encoding said sbp member or a polypeptide component thereof being contained within the host cell in a form that is capable of being packaged using said rgdp component, whereby the genetic material of the rgdp displaying an sbp member encodes said sbp member or a polypeptide component thereof, said sbp member or polypeptide component thereof being expressed from a phagemid as a capsid fusion, and a helper phage, or a plasmid expressing complementing phage genes, is used along with said capsid fusions to package the phagemid nucleic acid.

15. A method according to claim 14, wherein said capsid protein is absent, defective or conditionally defective in the helper phage.

16. A method according to any one of claims 13 to 15 wherein the host cell is a mutator strain which introduces genetic diversity into the sbp member nucleic acid.

17. A method according to any one of claims 9 to 16 wherein said library or genetically diverse population is obtained from:

(i) the repertoire of rearranged immunoglobulin genes of an animal immunised with complementary sbp member,
(ii) the repertoire of rearranged immunoglobulin genes of an animal not immunised with complementary sbp member,
(iii) a repertoire of an artificially rearranged immunoglobulin gene or genes,
(iv) a repertoire of an immunoglobulin homolog gene or genes, or
(v) a mixture of any of (i), (ii), (iii) and (iv).

18. A method according to any one of the preceding claims wherein said sbp member comprises a domain which is, or is homologous to, an immunoglobulin domain.

19. A method according to any one of the preceding claims

wherein the rgdp is a bacteriophage, the host is a bacterium, and said component of the rgdp is a capsid protein for the bacteriophage.

20. A method according to claim 19 wherein the phage is a filamentous phage.

21. A method according to claim 20 wherein the phage is selected from the class I phages fd, M13, f1, If1, 1ke, ZJ/Z, Ff and the class II phages Xf, Pf1 and Pf3.

22. A method according to claim 20 or claim 21 wherein said sbp member or polypeptide chain thereof is expressed as a fusion with the gene III capsid protein of phage fd or its counterpart in another filamentous phage.

23. A method according to claim 22 wherein said sbp member or polypeptide chain thereof is inserted in the N-terminal region of the mature capsid protein downstream of a secretory leader peptide.

24. A method according to any one of claims 19 to 23 wherein the host is E.coli.

25. A method according to any one of the preceding claims wherein nucleic acid encoding an sbp member polypeptide is linked downstream to a viral capsid protein through a suppressible translational stop codon.

26. A method according to any one of the preceding claims wherein the rgdps formed by said expression are selected or screened to provide an individual sbp member or a mixed population of said sbp members associated in their respective rgdps with nucleic acid encoding said sbp member or a polypeptide chain thereof.

27. A method according to claim 26 wherein the rgdps are selected by affinity with a member complementary to said sbp member.

28. A method according to claim 27 which comprises recovering any rgdps bound to said second member by washing with an eluant.

29. A method according to claim 28 wherein the eluant contains a molecule which competes with said rgdp for binding to the complementary sbp member.

30. A method according to any one of the claims 27 to 29 wherein the rgdp is applied to said complementary sbp member in the presence of a molecule which competes with said package for binding to said complementary sbp member.

31. A method according to any one of claims 26 to 30, wherein nucleic acid derived from a selected or screened rgdp is used to express said sbp member or a fragment or

derivative thereof in a recombinant host organism.

32. A method according to claim 31 wherein nucleic acid from one or more rgdps is taken and used to provide encoding nucleic acid in a further said method to obtain an individual sbp member or a mixed population of sbp members, or encoding nucleic acid therefor.

33. A method according to claim 31 or claim 32 wherein the expression end product is modified to produce a derivative thereof.

34. A method according to any one of claims 31, 32 and 33 wherein the expression end product or derivative thereof is used to prepare a therapeutic or prophylactic medicament or a diagnostic product.

35. Recombinant host cells harbouring a library of nucleic acid fragments comprising fragments encoding a genetically diverse population of a type of member of a specific binding pair (sbp), each sbp member or a polypeptide component thereof being expressed as a fusion with a component of a secretable replicable genetic display package (rgdp), so that said sbp members are displayed on surface of the rgdps in functional form and the genetic material of the rgdps encode the associated sbp member or a polypeptide component thereof.

36. Recombinant host cells according to claim 35, wherein said type of sbp member are immunoglobulins or immunoglobulin homologs, a first polypeptide chain of which is expressed as a said fusion with a component of the rgdp and a second polypeptide chain of which is expressed in free form and associates with the fused first polypeptide chain in the rgdp.

37. A helper phage whose genome lacks nucleic acid encoding one of its capsid proteins, or whose encoding nucleic acid therefor is conditionally defective, or which encodes said capsid protein in defective or conditionally defective form.

38. A bacterial host cell containing a filamentous phage genome defective for a capsid protein thereof and wherein the host cell is capable of expressing capsid protein complementing said defect such that infectious phage particles can be obtained therefrom.

39. A bacterial host cell according to claim 38 wherein said complementing capsid protein is expressed in said host from another vector contained therein.

40. A bacterial host cell according to claim 38 or claim 39 wherein the defective capsid protein is gene III of phage fd or its counterpart in another filamentous phage.

41. Recombinant E.coli TG1 M13KO7 gIII No. 3 (NCTC 12478).

42. A phage having the form of a replicable genetic display package displaying on its surface in functional form a member of a specific binding pair or a binding domain thereof.

43. A kit for use in carrying out a method according to any one of claims 1 to 34, said kit including:

(i) at least one vector having an origin of replication for single-stranded bacteriophage, a restriction site for insertion of nucleic acid encoding said sbp member or a polypeptide component thereof in the 5' end region of the mature coding sequence of a phage capsid protein, and with a secretory leader sequence upstream of said site which directs a fusion of the capsid protein and sbp polypeptide to the periplasmic space of a bacterial host; and

(ii) ancillary components required for carrying out the method.

Fig. 1.

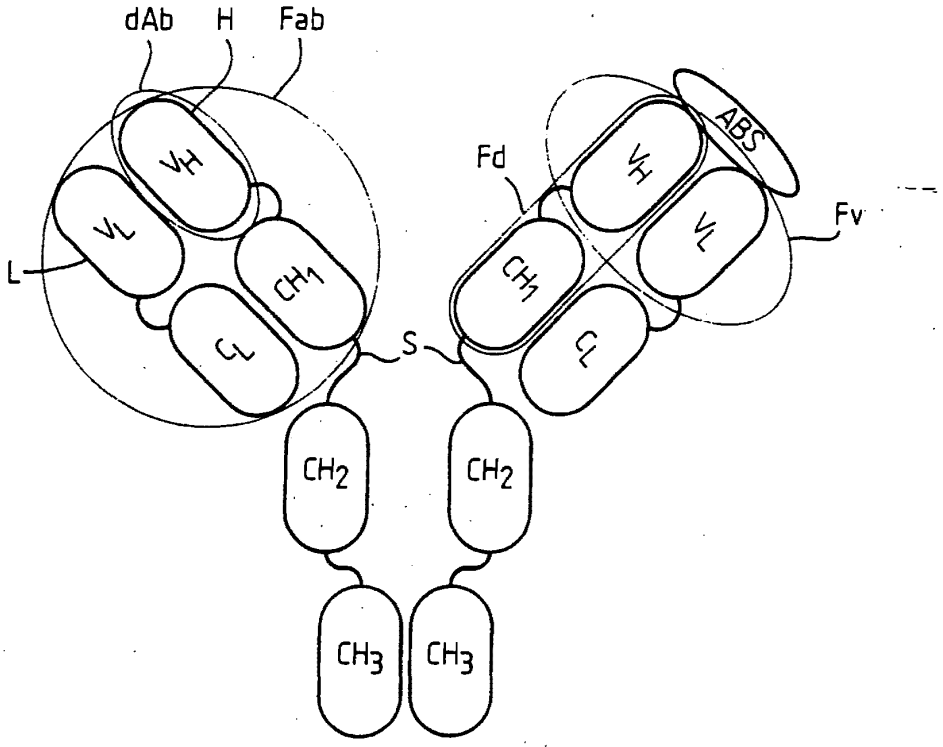


Fig. 2 (i)

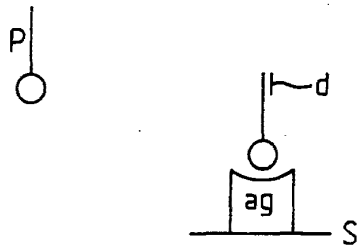
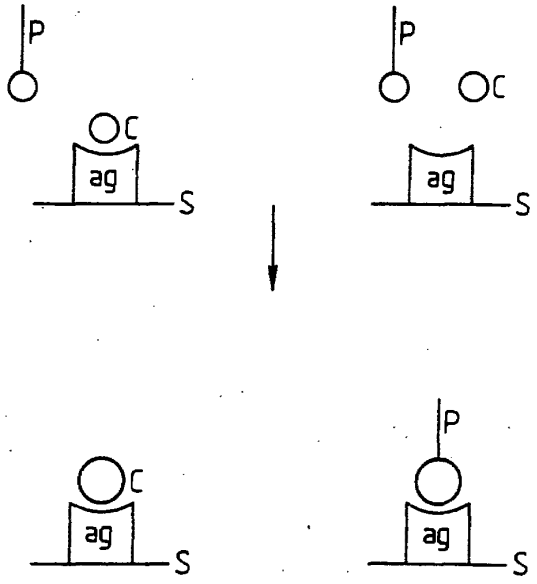


Fig. 2(ii)



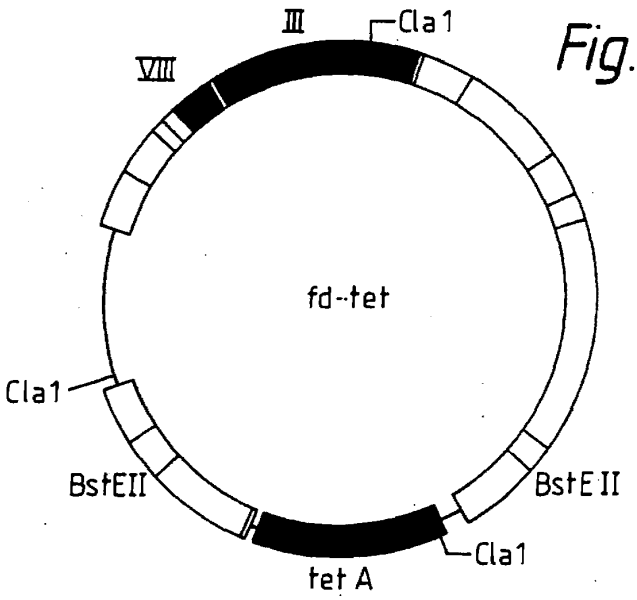


Fig.3.

fd - tet

⇓

cleave with BstEII

⇓

fill in with Klenow

⇓

re-ligate

↓

FDT δ Bst

⇓

in vitro mutagenesis (oligo 1)

↓

FDTPs/Bs

⇓

in vitro mutagenesis (oligo 2)

↓

FDTPs/Xh

Fig. 4.1

(1653)
Oligo 1 ACA ACT TTC AAC AGT TGA GGA GAC GGT GAC CGT AAG CTT CTG CAG TTG GAC CTG AGC
GGA GTG AGA ATA (1620)

(1653)
Oligo 2 ACA ACT TTC AAC AGT TTC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG

(1704)
Oligo 3 GTC GTC TTT CCA GAC GTT AGT

Fig. 4.2

GENE III — GENE III

SIGNAL
CLEAVAGE SITE

(1624) (1650)

A TCT CAC TCC GCT GAA ACT GTT GAA AGT

Q V Q L Q V T V S S

B TCT CAC TCC GCT CAG GTC CAA CTG CAG AAG CTT ACG GTC ACC GTC TCC TCA ACT GTT GAA AGT
PstI BstEII

Q V Q L Q L E I K R

C TCT CAC TCC GCT CAG GTC CAA CTG CAG GAG CTC GAG ATC AAA CGG GAA ACT GTT GAA AGT
PstI XhoI

Fig. 5.

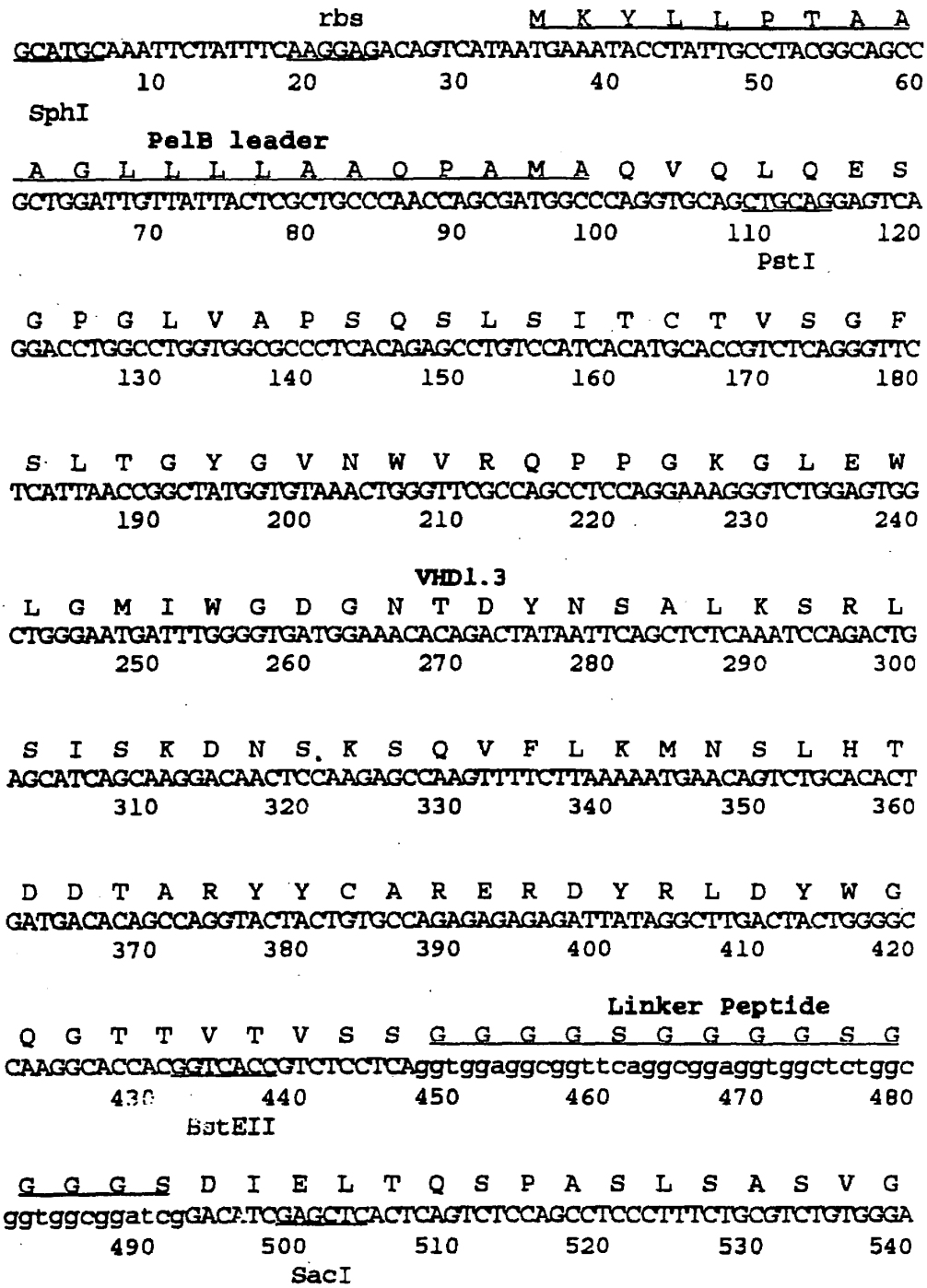


Fig. 5 cont.

```
 E  T  V  T  I  T  C  R  A  S  G  N  I  H  N  Y  L  A  W  Y
GAAACTGTCACCATCACATGTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTAT
      550        560        570        580        590        600

 Q  Q  K  Q  G  K  S  P  Q  L  L  V  Y  Y  T  T  T  L  A  D
CAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAACCTTAGCAGAT
      610        620        630        640        650        660

                                VKD1.3
 G  V  P  S  R  F  S  G  S  G  S  G  T  Q  Y  S  L  K  I  N
GGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACAATATTCTCTCAAGATCAAC
      670        680        690        700        710        720

 S  L  Q  P  E  D  F  G  S  Y  Y  C  Q  H  F  W  S  T  P  R
AGCCTGCAACCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTTGGAGTACTCCTCGG
      730        740        750        760        770        780

                                                Myc Tag (TAG1)
 T  F  G  G  G  T  K  L  E  I  K  R  E  Q  K  L  I  S  E  E
ACGTTCGGTGGAGGGACCAAGCTCGAGATCAAACGGGAACAAAAACTCATCTCAGAAGAG
      790        800        810        820        830        840
                      XhoI

 D  L  N  *  *
GATCTGAATTAATAATGATCAAACGGTAATAAGGATCCAGCTCGAATTC
      850        860        870        880
                                          EcoRI
```

Fig.6.

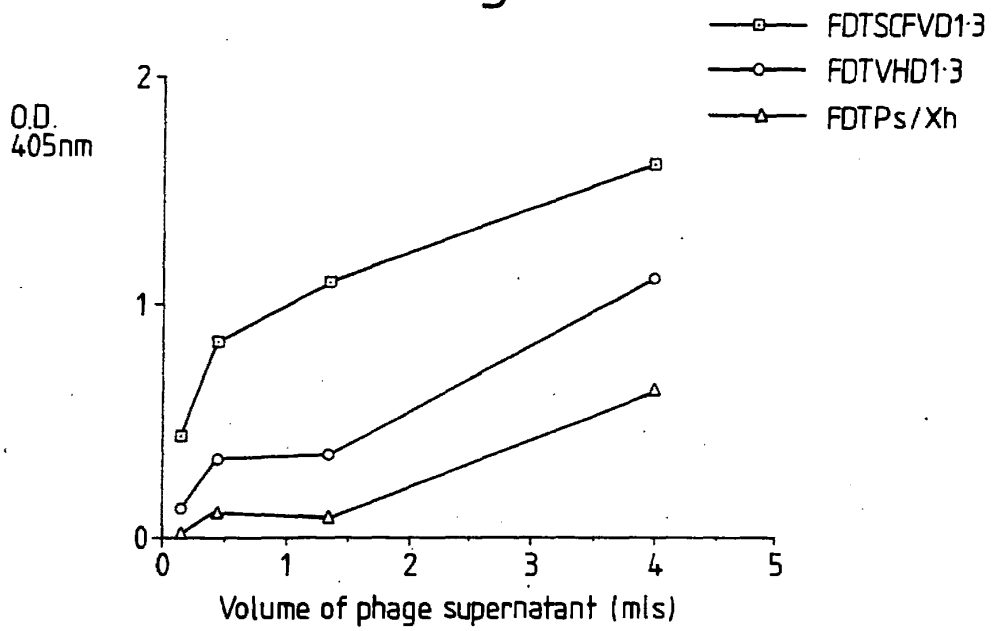


Fig.7.

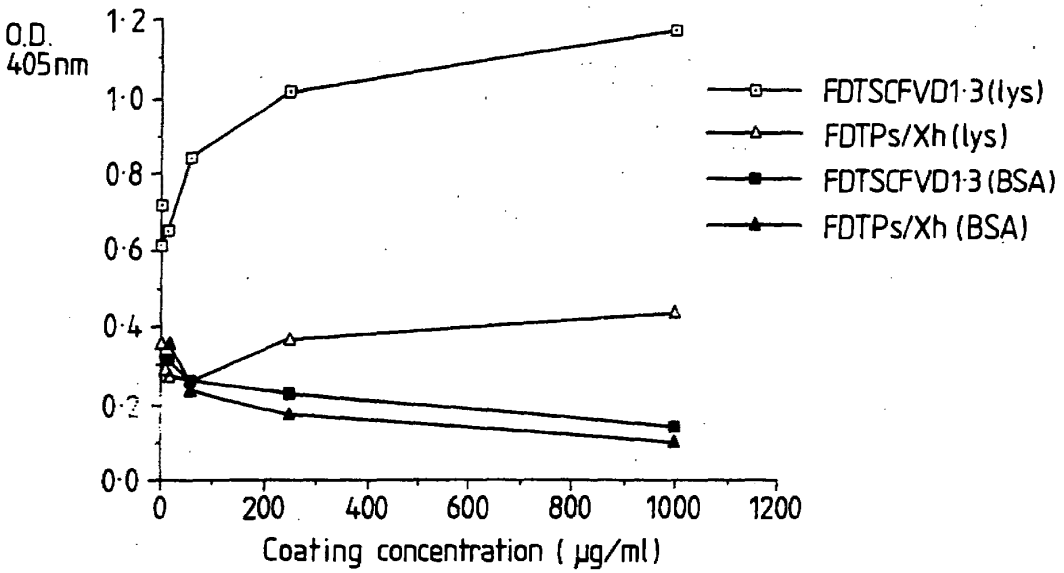


Fig. 8.

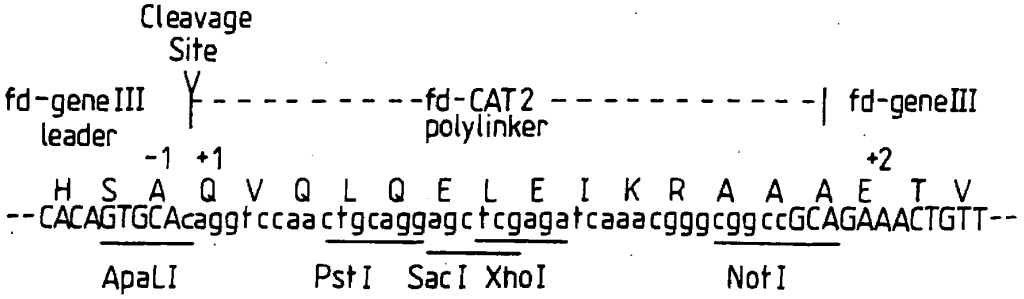


Fig. 9.

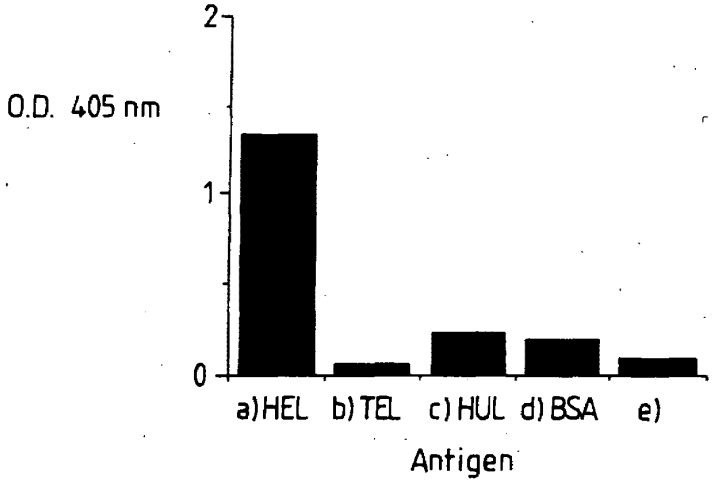


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Fig.10.

```
                                                    M  K  Y  L  L  P  T  A  A
GCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCC
        10         20         30         40         50         60

 A  G  L  L  L  L  A  A  Q  P  A  M  A  Q  V  Q  L  Q  E  S
GCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCCAGGTGCAGCTGCAGGAGTCA
        70         80         90        100        110        120

 G  P  G  L  V  A  P  S  Q  S  L  S  I  T  C  T  V  S  G  F
GGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTC
       130        140        150        160        170        180

 S  L  T  G  Y  G  V  N  W  V  R  Q  P  P  G  K  G  L  E  W
TCATTAACCGGCTATGGTGTAAACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGG
       190        200        210        220        230        240

 L  G  M  I  W  G  D  G  N  T  D  Y  N  S  A  L  K  S  R  L
CTGGGAATGATTTGGGGTGATGGAAACACAGACTATAATTCAGCTCTCAAATCCAGACTG
       250        260        270        280        290        300

 S  I  S  K  D  N  S  K  S  Q  V  F  L  K  M  N  S  L  H  T
AGCATCAGCAAGGACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCACACT
       310        320        330        340        350        360

 D  D  T  A  R  Y  Y  C  A  R  E  R  D  Y  R  L  D  Y  W  G
GATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGC
       370        380        390        400        410        420

 Q  G  T  T  V  T  V  S  S  A  S  T  K  G  P  S  V  F  P  L
CAAGGCACCACGGTCACCGTCTCCTCAGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTG
       430        440        450        460        470        480

 A  P  S  S  K  S  T  S  G  G  T  A  A  L  G  C  L  V  K  D
GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGAC
       490        500        510        520        530        540
```

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Fig.10 cont.(1)

```
 Y  F  P  E  P  V  T  V  S  W  N  S  G  A  L  T  S  G  V  H
TACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCAC
      550        560        570        580        590        600

 T  F  P  A  V  L  Q  S  S  G  L  Y  S  L  S  S  V  V  T  V
ACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTG
      610        620        630        640        650        660

 P  S  S  S  L  G  T  Q  T  Y  I  C  N  V  N  H  K  P  S  N
CCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAAC
      670        680        690        700        710        720

 T  K  V  D  K  K  V  E  P  K  S  S  *  *
ACCAAGGTCGACAAGAAAGTTGAGCCCAAATCTTCATAATAACCCGGGAGCTTGCATGCA
      730        740        750        760        770        780

                              M  K  Y  L  L  P  T  A  A  A  G  L
AATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGAT
      790        800        810        820        830        840

    L  L  L  A  A  Q  P  A  M  A  D  I  E  L  T  Q  S  P  A  S
TGTTATTACTGGCTGCCCAACCAGCGATGGCCGACATCGAGCTCACCCAGTCTCCAGCCT
      850        860        870        880        890        900

    L  S  A  S  V  G  E  T  V  T  I  T  C  R  A  S  G  N  I  H
CCCTTTCTGCGTCTGTGGGAGAAACTGTCACCATCACATGTCGAGCAAGTGGGAATATTC
      910        920        930        940        950        960

    N  Y  L  A  W  Y  Q  Q  K  Q  G  K  S  P  Q  L  L  V  Y  Y
ACAATTATTTAGCATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATT
      970        980        990       1000       1010       1020
```

Fig. 10 cont. (2)

```
   T  T  T  L  A  D  G  V  P  S  R  F  S  G  S  G  S  G  T  Q
ATACAACAACCTTAGCAGATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACAC
      1030       1040       1050       1060       1070       1080

   Y  S  L  K  I  N  S  L  Q  P  E  D  F  G  S  Y  Y  C  Q  H
AATATTCTCTCAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAAC
      1090       1100       1110       1120       1130       1140

   F  W  S  T  P  R  T  F  G  G  G  T  K  L  E  I  K  R  T  V
ATTTTTGGAGTACTCCTCGGACGTTCGGTGGAGGCACCAAGCTCGAGATCAAACGGACTG
      1150       1160       1170       1180       1190       1200

   A  A  P  S  V  F  I  F  P  P  S  D  E  Q  L  K  S  G  T  A
TGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTG
      1210       1220       1230       1240       1250       1260

   S  V  V  C  L  L  N  N  F  Y  P  R  E  A  K  V  Q  W  K  V
CCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGG
      1270       1280       1290       1300       1310       1320

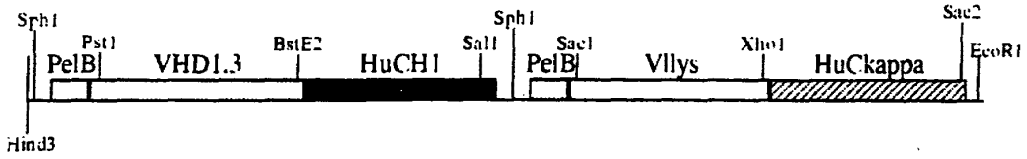
   D  N  A  L  Q  S  G  N  S  Q  E  S  V  T  E  Q  D  S  K  D
TGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGG
      1330       1340       1350       1360       1370       1380

   S  T  Y  S  L  S  S  T  L  T  L  S  K  A  D  Y  E  K  H  K
ACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACA
      1390       1400       1410       1420       1430       1440

   V  Y  A  C  E  V  T  H  Q  G  L  S  S  P  V  T  K  S  F  N
AAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCA
      1450       1460       1470       1480       1490       1500

   R  G  E  S  *  *
ACCGCGGAGAGTCATAGTAAGAATTC
      1510       1520
```

Fig.10 cont.(3)



FabD1.3 in pUC19

Fig. 11.

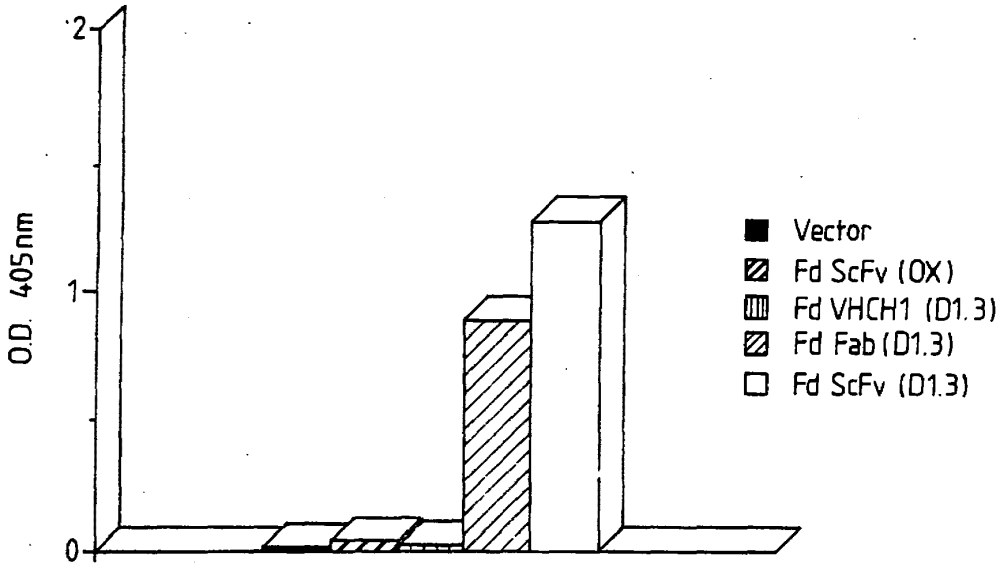


Fig. 12.

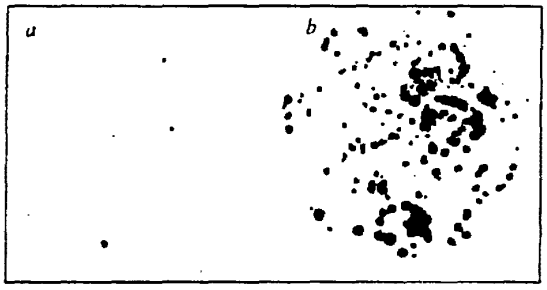


Fig.13.

```
 Q   V   Q   L   Q   E   S   G   G   G   L   V   Q   P   G   G
CAG GTG CAG CTG CAG GAG TCA GGA GGA GGC TTG GTA CAG CCT GGG GGT
            PstI
 S   L   R   L   S   C   A   T   S   G   F   T   F   S   N   Y
TCT CTG AGA CTC TCC TGT GCA ACT TCT GGG TTC ACC TTC AGT AAT TAC

 Y   M   G   W   V   R   Q   P   P   G   K   A   L   E   W   L
TAC ATG GGC TGG GTC CGC CAG CCT CCA GGA AAG GCA CTT GAG TGG TTG

 G   S   V   R   N   K   V   N   G   Y   T   T   E   Y   S   A
GGT TCT GTT AGA AAC AAA GTT AAT GGT TAC ACA ACA GAG TAC AGT GCA

 S   V   K   G   R   F   T   I   S   R   D   N   F   Q   S   I
TCT GTG AAG GGG CGG TTC ACC ATC TCC AGA GAT AAT TTC CAA AGC ATC

 L   Y   L   Q   I   N   T   L   R   T   E   D   S   A   T   Y
CTC TAT CTT CAA ATA AAC ACC CTG AGA ACT GAG GAC AGT GCC ACT TAT

 Y   C   A   R   G   Y   D   Y   G   A   W   F   A   Y   W   G
TAC TGT GCA AGA GGC TAT GAT TAC GGG GCC TGG TTT GCT TAC TGG GGC

 Q   G   T       L   V   T   v   s   s   g g g g s g g g g s
CAA GGG ACC   CTG GTC ACC gtc tcc tca ggtggaggcggttcaggcggaggtggctct
                BstEII
 g g g g s d   i   E   L   T   Q   T   P   L   S   L   P   V
ggcggtggcggatcggac atc GAG CTC ACC CAA ACT CCA CTC TCC CTG CCT GTC
                      SacI
 S   L   G   D   Q   A   S   I   S   C   R   S   S   Q   S   I
AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT

 V   H   S   N   G   N   T   Y   L   E   W   Y   L   Q   K   P
GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA CCA
                                            PstI
 G   Q   S   P   K   L   L   I   Y   K   V   S   N   R   F   S
GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT

 G   V   P   D   R   F   S   G   S   G   S   G   T   D   F   T
GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCG GGG ACA GAT TTC ACA

 L   K   I   S   R   V   E   A   E   D   L   G   V   Y   Y   C
CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC

 F   Q   G   S   H   V   P   Y   T   F   G   G   G   T   K   L
TTT CAA GGT TCA CAT GTT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTC

 E   I   K   R
GAG ATC AAA CGG
XhoI
```

Fig. 14.

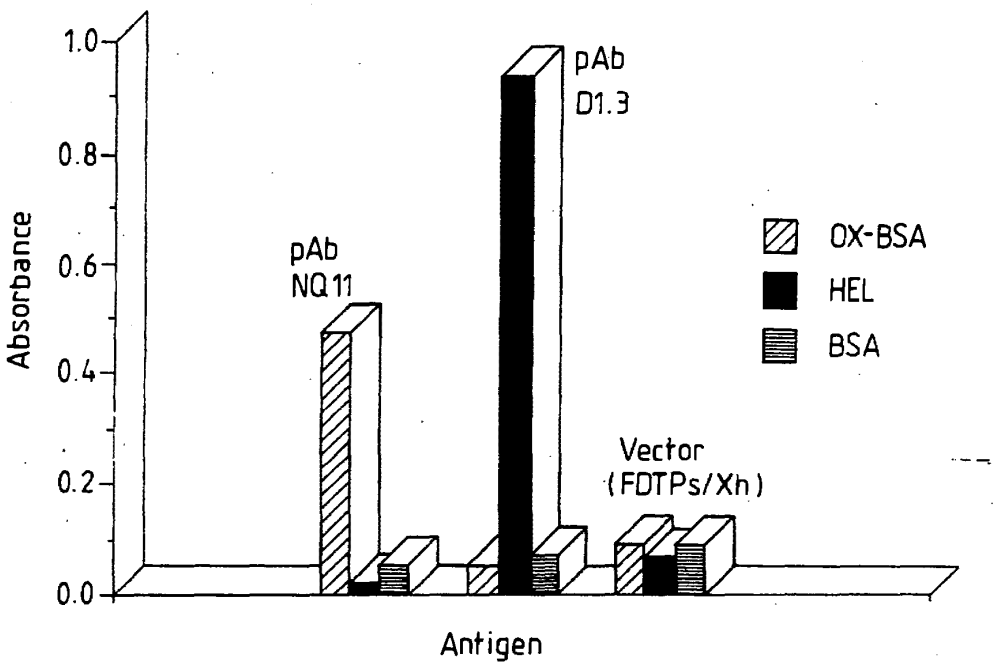


Fig. 15.

5' END

R T P E M P V L

TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG

ApaL1

3' END

K A A L G L K

AAA GCC GCT CTG GGG CTG AAA GCG GCC GCA GAA ACT GTT GAA AGT etc.

Not I

Fig.16(1)

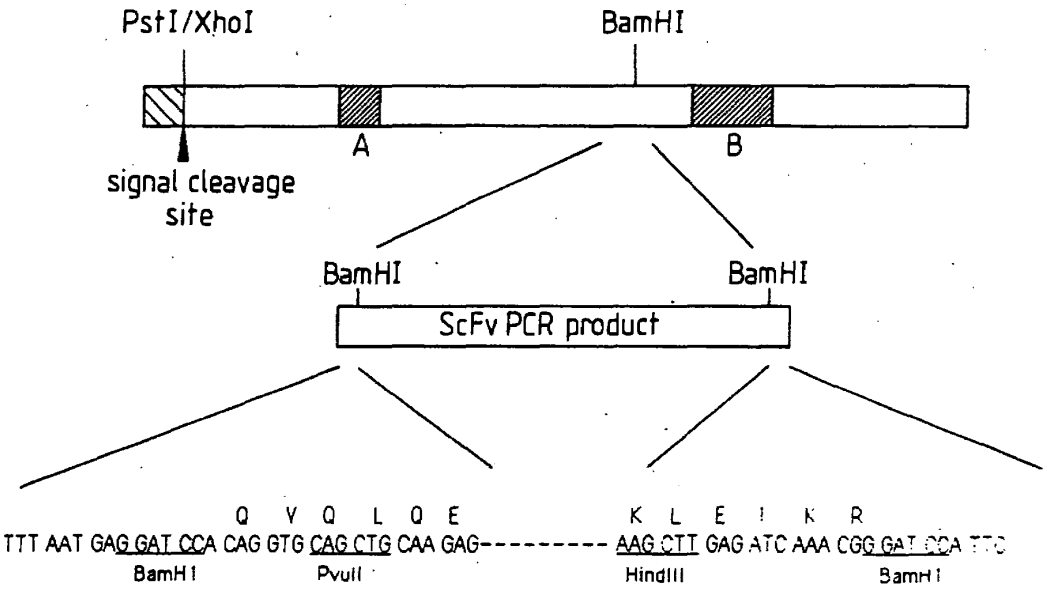


Fig.16(2)

A

(1834) 5' GAG GGT GGT GGC TCT
" " "C " "
" " "C " "
" " "C " ACT 3' (1839)

B

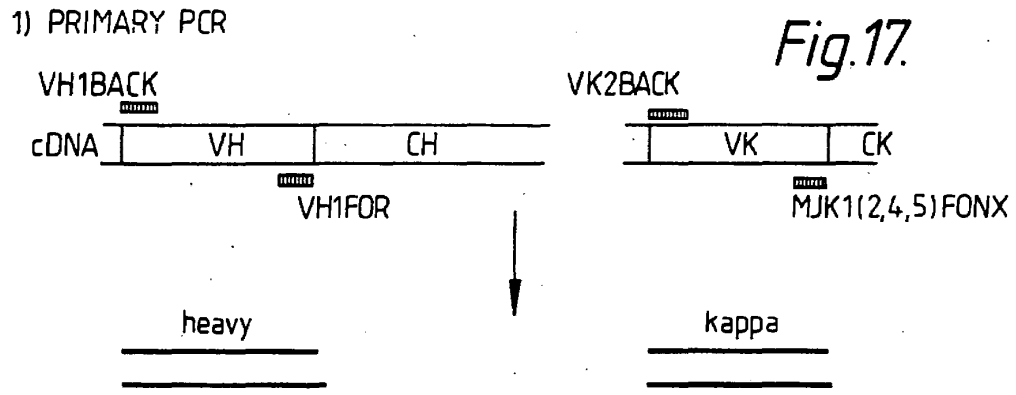
(2284) 5' - GGC GGC GGC TCT
- GGT GGT GGT "
- " GGC GGC "
GAG " " GGC "
" " " GGT "
" " " GGC "
" " " GGT "
- " " GGC " 3' (2379)

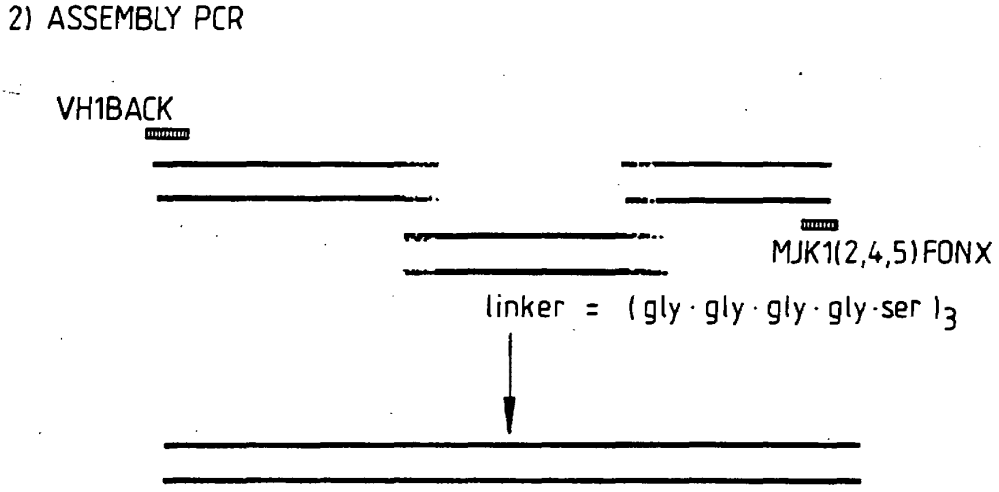
Reverse complement of mutagenic oligo G3Bamlink

T
5' GAG GGT GGC GGA TCC

T
GAG GGT GGC GG 3'

Fig.17.





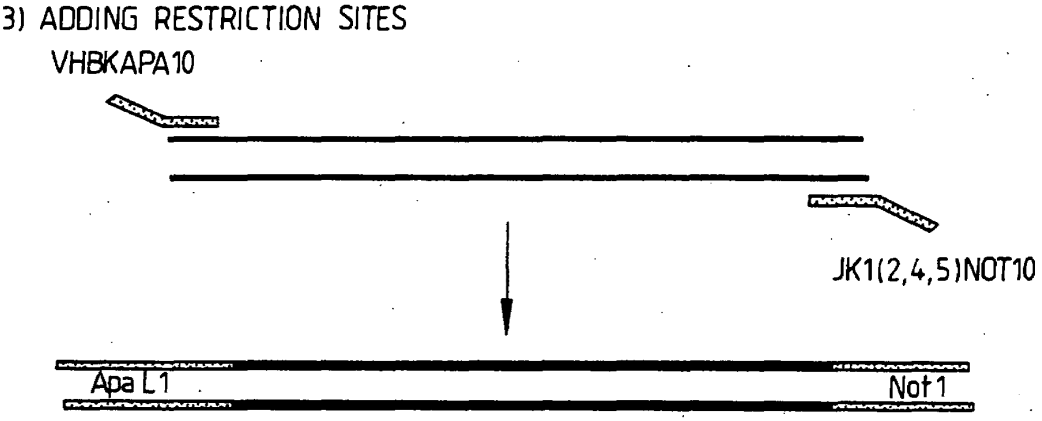


Fig. 18.

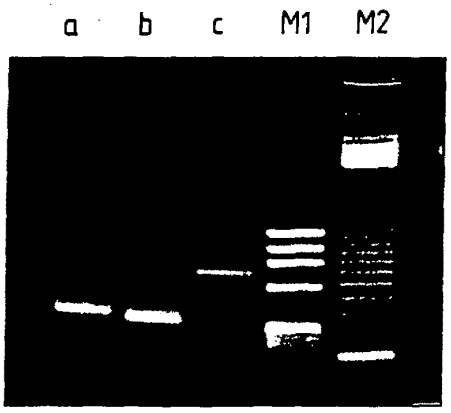


Fig.19.

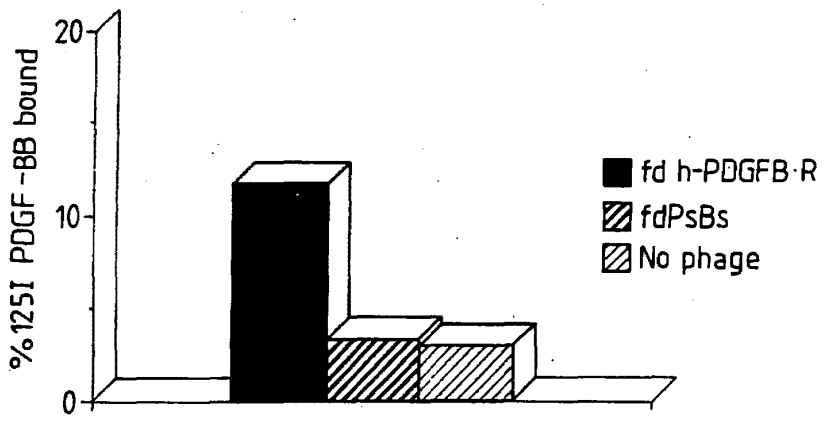


Fig.20.

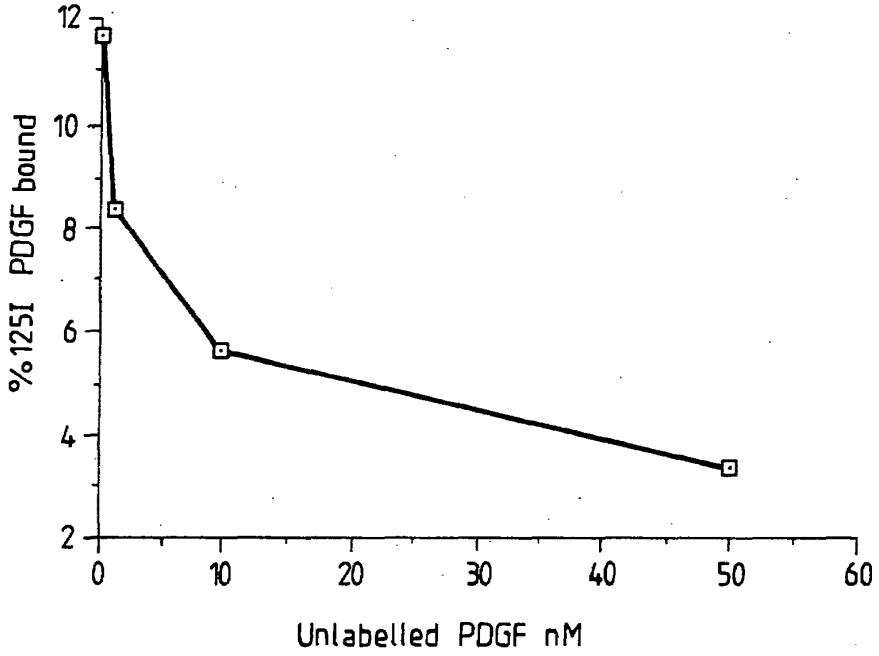


Fig.21.

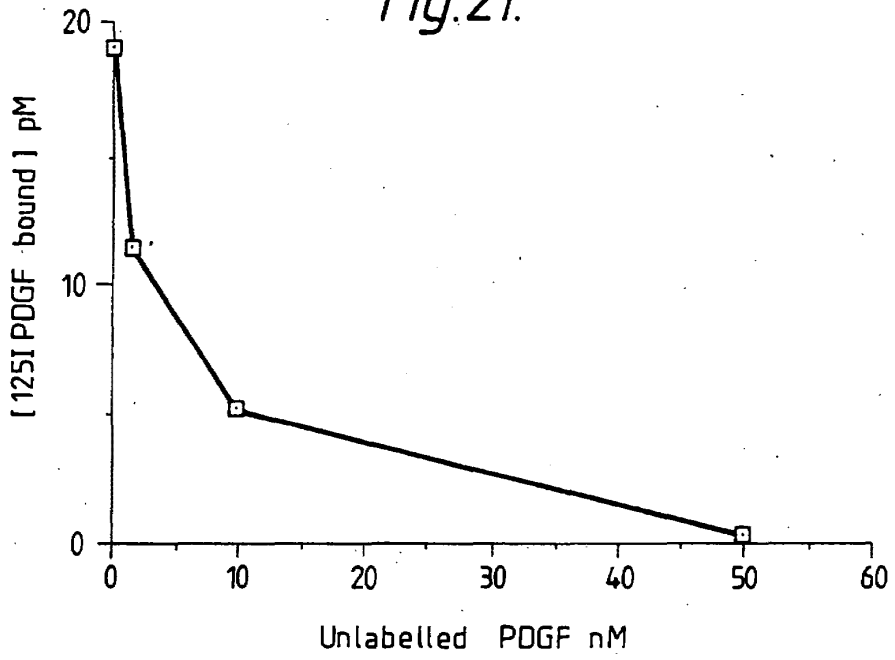


Fig.22.

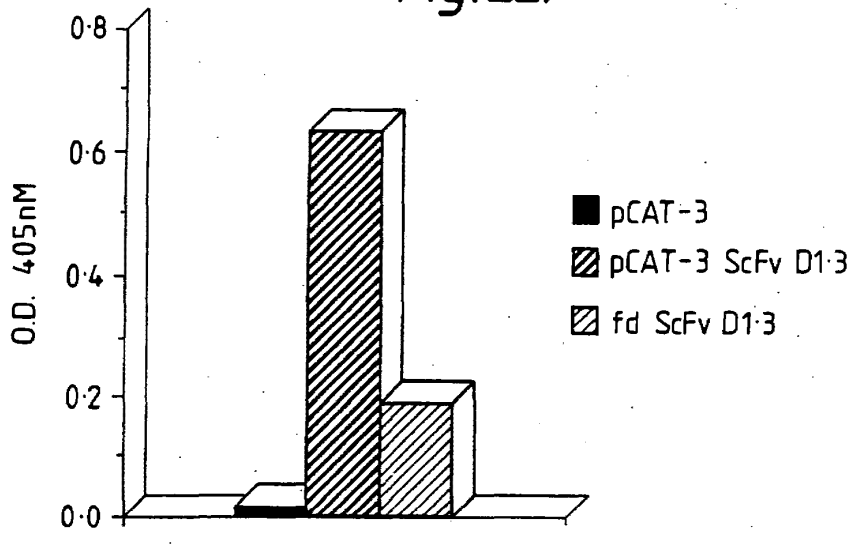
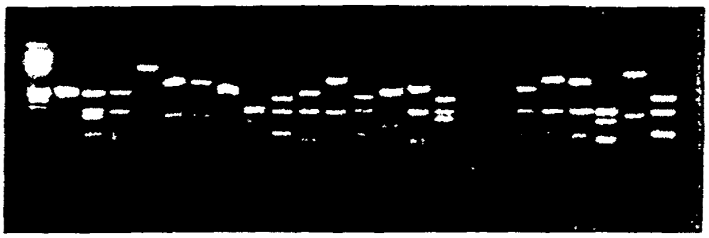


Fig.23.

d

M



M

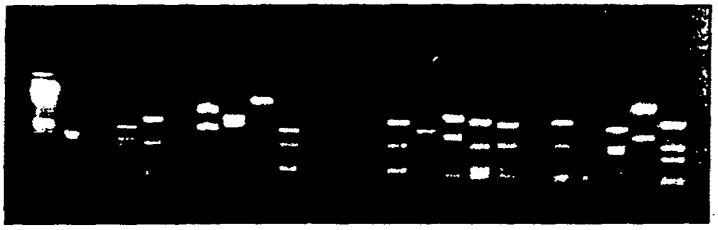


Fig. 24.

VH sequences

from combinatorial library:

		CDR1		CDR2		CDR3			
A	QVQLQQSGAELARPGASVKMSCKASGYTFT	SYTMH	WVKQRPGQGLEWIG	YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAI	RYGAY	WGQGTTVTVSS	x4	1	
B	QVKLQQSGAELAKPGASVKMSCKASGYTFT	RDWMH	WLKQRPGQGLEWIG	YIIPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	HYGLY	WGQGTTVTVSS	x9	1	
C	QVQLQQSGPELVKPGASVKMSCKASGYTFT	SYVMH	WVKQKPGQGLEWIG	YINPYNDGTKYNEKFKG	KATLTSDKSSSTAYMELSSLTSEDSAVYYCAI	YRSPPY	WGQGTTVTVSS	x3	1	
D	QVQLQQSGPELVKPGASVKISCKASGYSFT	GYFMN	WVKQSHGKSLEWIG	RINPYNGDTFYNQKFKG	KATLTVDKSSSTAHMELLSLTSEDSAVYYCVG	ITTRFAY	WGQGTTVTVSS	x1	1	
E	QVQLQESGPGLVAPSQSLSITCTVSGFSLT	SYGVH	WVRQPPGKGLEWLG	VIWAGGSTNYNSALMS	RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAR	DRGDY	WGQGTTVTVSS		3	VHox1
F	QVQLQQSGPELAKPGASVKMSCKASGYTFT	SYLMH	WVKQRPGQGLKWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	
G	QVKLQQSGAELVRPGASVKLSCKASGYTFT	RYLMH	WVKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	EATLTADKSSNTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	
H	QVQLQQSGPELMKPGASVKISCKASGYSFS	RHYMH	WVKQSHGKSLEWIG	YIAPFNGGTTYNQKFKG	KATLTVDRSSSTAYMHLSSLTSEDSAVYYCAT	DYGRD	WGQGTTVTVSS		1	

from hierarchical library VH-rep x Vκ-d:

I	QVKLQQSGPELARPGVSVKMSCKASGYTFT	SYAMH	WVKQSQSKSLEWIG	VISTYNGNTNYNQKFKG	KATMTVDKSSSTAYMSLARLTSEDSAIYYCAR	DYGDY	WGQGTTVTVSS		1	
J	QVKLQQSGAELARPGASVKMSCKASGYTFT	RYTMH	WVKQRPGQGLEWIG	YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DRGAY	WGQGTTVTVSS		1	
K	QVKLQQSGAELAKPGASVKMSCKASGYTFT	RDWMH	WVKQRPGQGLEWIG	YIIPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	HYGLY	WGQGTTVTVSS	x3	1	
L	QVQLQQSGLELAKPGASVKMSCKASGYTFT	HYLMH	WVKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS	x2	1	
M	QVKLQQSGAELAKPGASVKMSCKASGYTFT	HYWMH	WVKQRPGQGLEWIG	YIIPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSDDSAVYYCAR	DYGYF	WGQGTTVTVSS		1	
N	QVQLQQSGAELVKPGASVKLSCKTSGYTFT	SYTMN	WVKQRPGQGLEWIG	YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	
O	QVQLQQSGAELAKPGASVKMSCEASGYTFT	SHLMH	WVKQRPGQGLEWIG	YINPRTGYTEYNQKFKD	KATFTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGAY	WGQGTTVTVSS		1	
P	QVKLQQSGAELAKPGASVKMSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	
Q	QVKLQQSGAELAKPGASVKMSCKATGYTFT	SYLMH	WVKQRPGQGLEWIG	YIIPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	
R	QVQLQQSGAELAKPGASVKMSCKASGYTFT	SYVMH	WVKQRPGQGLEWIG	YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	NYGIY	WGQGTTVTVSS		1	
S	QVQLQQSGAELAKPGASVKMSCKASGYTFT	TFLMH	WLKQRPGQGLEWIG	YIIPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS	x2	1	
T	QVKLQQSGAELARPGASVKMSCKASGYTFT	SYTMH	WVKQRPGQGLDWIG	YIIPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS	x6	1	
U	QVKLQQSGAELAKPGASVKMSCKASGYTFT	SYTMH	WVKQRPGQGLEWIG	YIIPTTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	
B	*QVKLQQSGAELAKPGASVKMSCKASGYTFT*	*RDWMH*	*WLKQRPGQGLEWIG*	*YINPSTGYTEYNQKFKD*	*KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR*	*NYGLY*	*WGQGTTVTVSS*			

Fig. 24 cont.

Vκ sequences

from combinatorial library:

		CDR1		CDR2		CDR3				
a	DIELTQSPSSLSASLGERVSLTC	RASQEISGYLS	WLQQKPDGSIKRLIY	AASTLES	GVPKRFSGSRSGSDYSLTISSLESEDFADYYC	LQYASYPT	FGAGTKLEIKRA	x3	V	ox-like
b	DIELTQSPAIMSASPGEKVTMTC	RASSSVSSSYLH	WYQQKSGASPKVWIY	STSNLAS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGAGTKLEIKRA	x3	IV	ox-like
c	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPLT	FGAGTKLEIKRA	x2	IV	ox-like
d	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WFQQKPGFSPKLLIS	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSTIPFT	FGSGTKLEIKRA	x9	IV	ox-like
e	DIELTQSPAIMSASPGEKVTITC	SASSSVNYMH	WFQQKPGTSPKLWIY	STSNLAS	GVPTRFSGSGSGTSYSLTISRMEAEDAATYYC	QQRSSYPPT	FGSGTKLEIKRA	x4	VI	ox-like?
f	DIELTQSPAIMSAFPGEKVTMTC	SASSSVSYMH	WYQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQFSSNPLT	FGAGTKLELKRA		VI	Vkox1
g	DIELTQSPAIMSASPGEKVTMTC	SASSSINYMH	WYQQKPGASPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	HQRISYPWT	FGGGTKLEIKRA		VI	ox-like?

from hierarchical library VH-B x Vκ-rep:

h	DIELTQSPAIMSASPGEKVTMTC	SASSSVSYMH	WYQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQWSSNPLT	FGAGTKLEIKRA	x4	IV/VI	Vkox1
i	DIELTQSPAIMSASPGEKVTITC	SASSSVSYIH	WFQQKPGTSPKLWIY	STSNLAS	GVPARFSGSGSGTSYSLTISRMEAEDAATYYC	QQYHSYPLT	FGAGTKLEIKRA		V	ox-like?
j	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WFQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPLT	FGGGTKLEIKRA		V	ox-like
k	DIELTQSPTTMAASPGDMITITC	SATSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPPRFSGSGSGTSYSLTIGAMEAEDVATYYC	QQGSSIPYT	FGAGTKLEIKRA		V	ox-like
l	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPYT	FGGGTKLEIKRA		V	ox-like
m	DIELTQSPTTMAASPGEKITITC	SASSSISSNHLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSGIPYT	FGGGTKLEIKRA		V	ox-like
n	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPFT	FGGGTKLEIKRA		V	ox-like
o	DIELTQSPAIMAASPGEKITITC	SASSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPYT	FGGGTKLEIKRA	x2	V	ox-like
p	DIELTQSPAIMSASPGEKVTMTC	SASSSVSYMH	WYQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDVATYYC	QQWSSNPLT	FGAGTKLEIKRA	x2	IV/VI	Vkox1
q	DIELTQSPAIMSASPGDKVTLTC	SASSSVRYVN	WFQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQWTSNPPT	FGGGTKLEIKRA		IV/VI	Vkox1
r	DIELTQSPAIMSASPGEKVTMTC	SASSSVSYMH	WYQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQWSTNALT	FGAGTKLEIKRA		IV/VI	Vkox1
s	DIELTQSPAIMSASPGEKVTMTC	RASSSVTSSYLH	WYQQKSGASPKLWVY	STSNLAS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGAGTKLEIKRA		IV/VI	ox-like
t	DIELTQSPAIMSASPGEKVTMTC	RASSSVSSSYLH	WYQQKSGASPKLWIY	STSNLAS	GVPARFSGSGSGTSYSLTISRMEAEDAATYYC	QQRSSYPLT	FGAGTKLEIKRA		IV/VI	ox-like
u	DIELTQSPAIMSASPGEKVTMTC	RASSSVSSSYLH	WYQQKSGASPKLWIY	STSNLAS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGAGTKLEIKRA		IV/VI	ox-like
v	DIELTQSPAIMSASPGEKVTMTC	RASSSVSSSYLH	WFQQKSGASPKLWIY	STSNLPS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGGGTKLEIKRA		IV/VI	ox-like
c	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPLT	FGAGTKLEIKRA	x3		

Fig. 25.

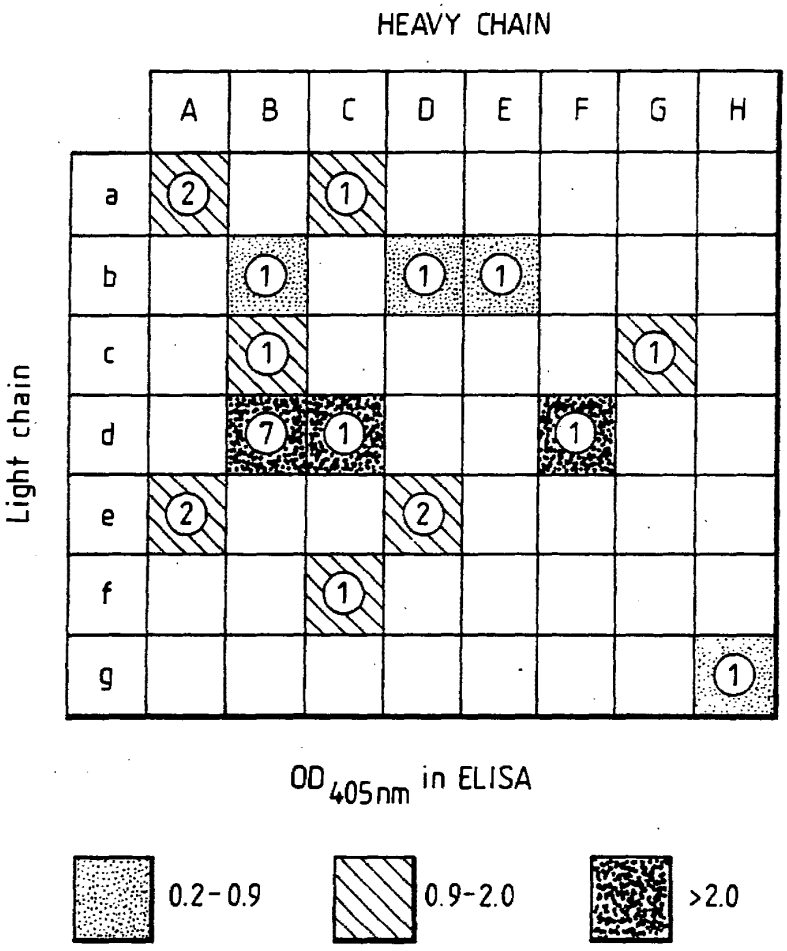


Fig.26(a)

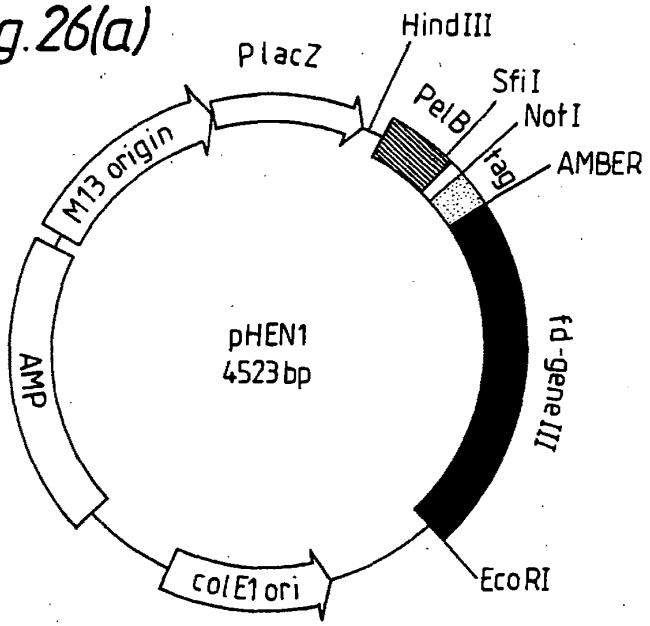
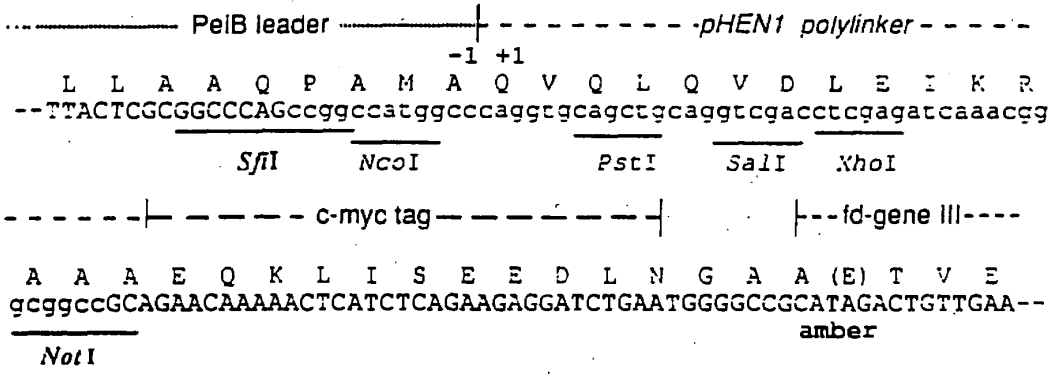


Fig.26(b)



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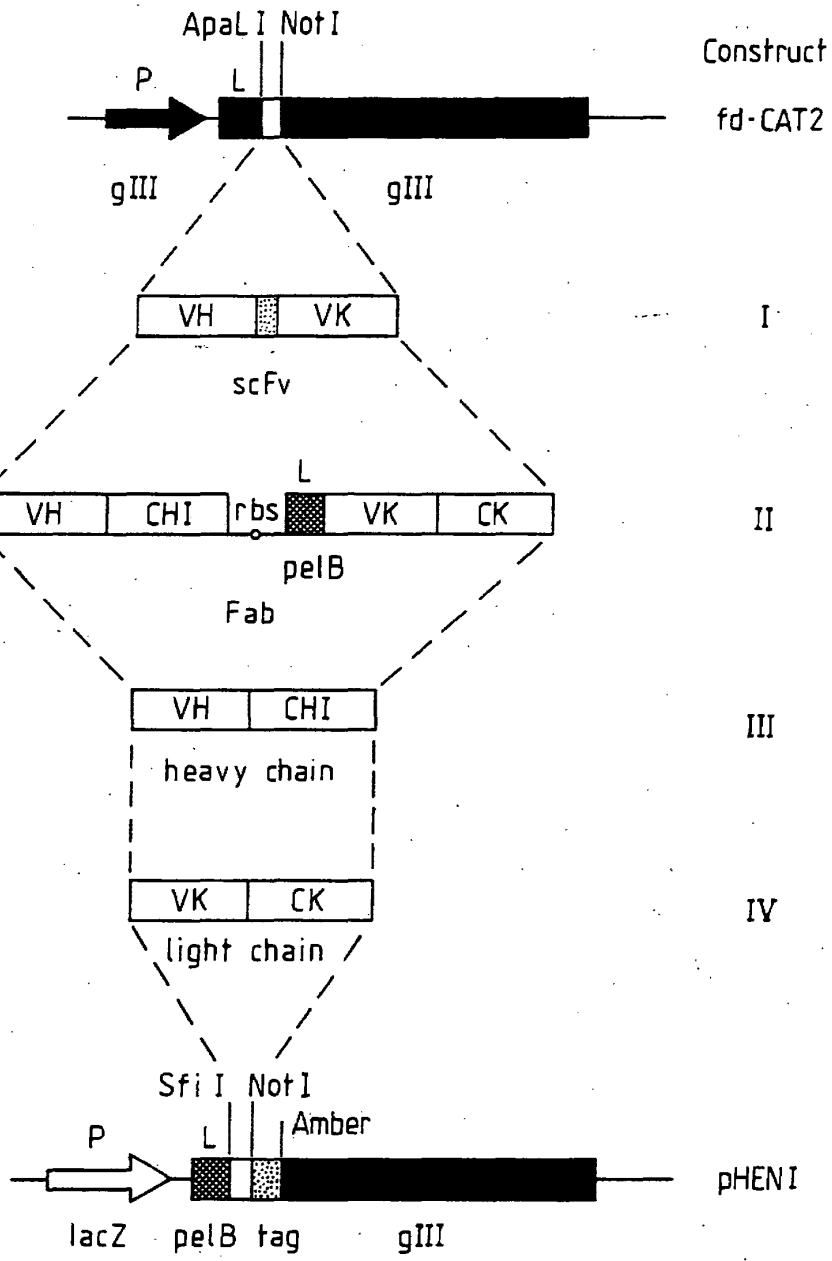

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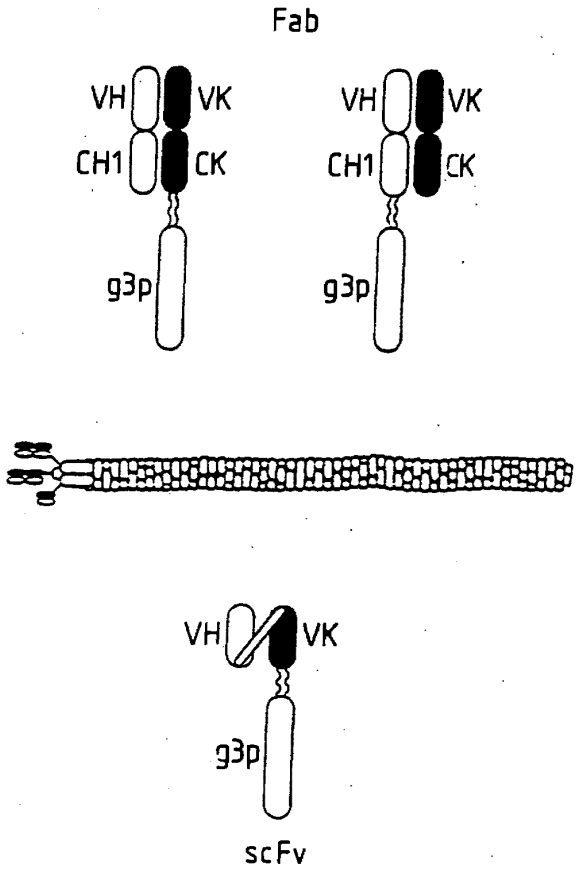

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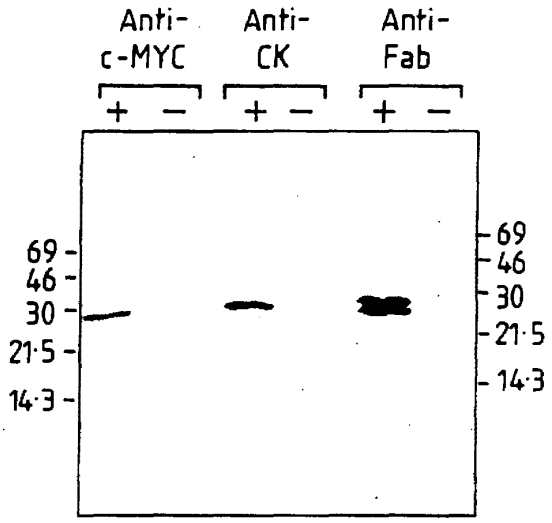

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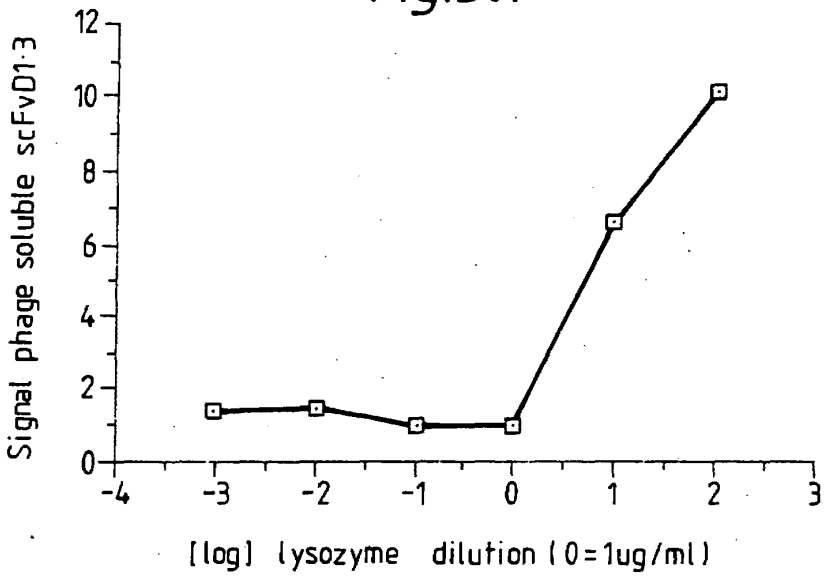
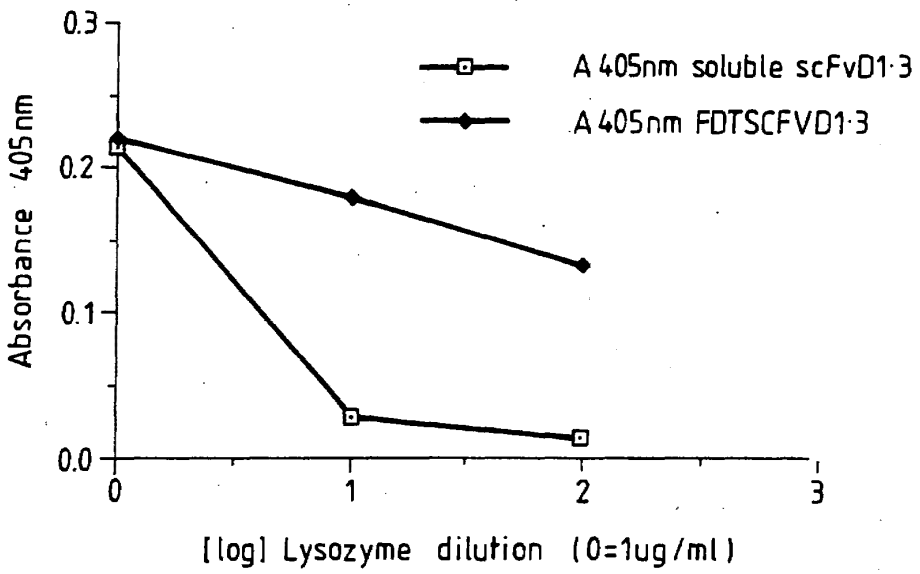


Fig.31.



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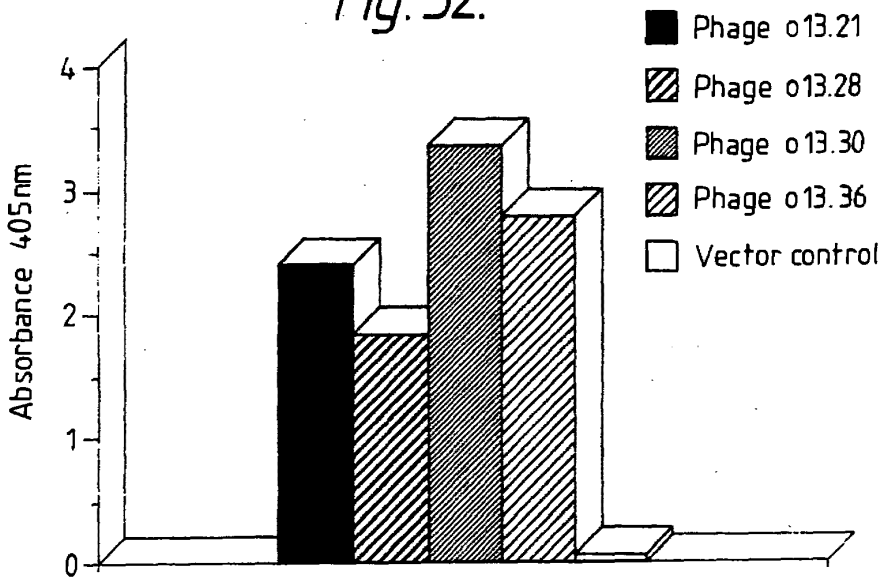
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1
2
3
4
Phage o13.21
Phage o13.28
Phage o13.30
Phage o13.36
Vector control

Fig. 33.

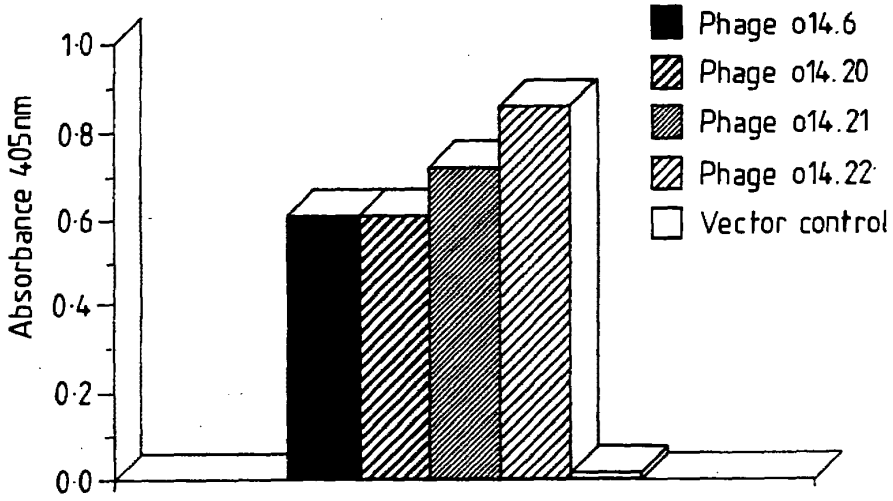

Absorbance 405nm
0·0
0·2
0·4
0·6
0·8
1·0
Phage o14.6
Phage o14.20
Phage o14.21
Phage o14.22
Vector control

Fig. 34.

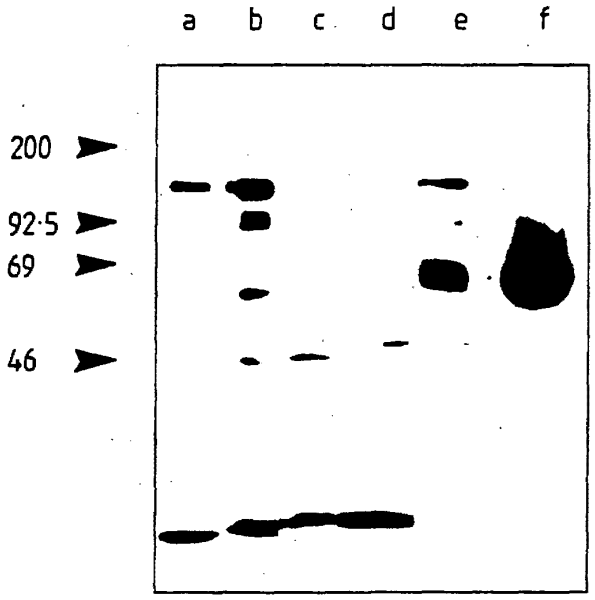
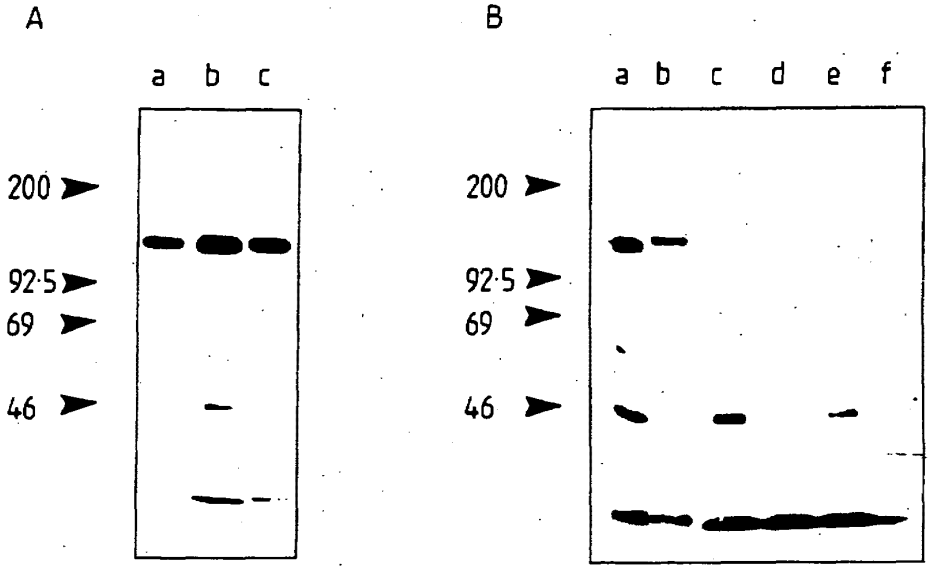


Fig. 35.



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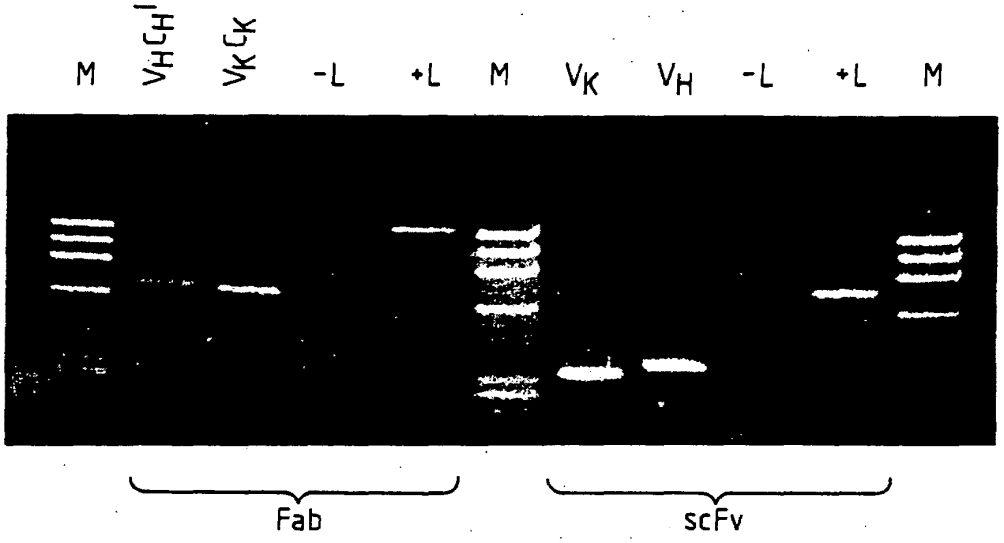


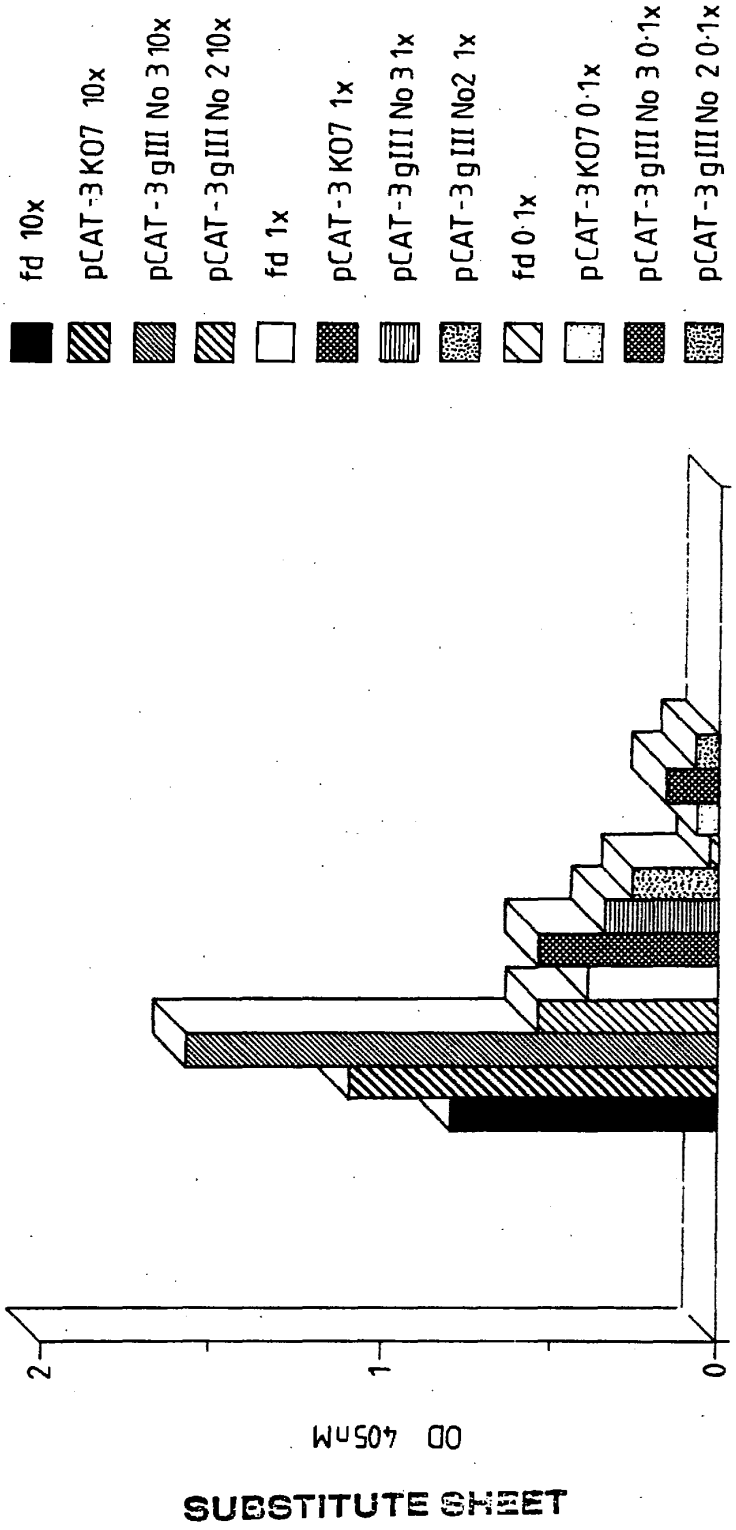
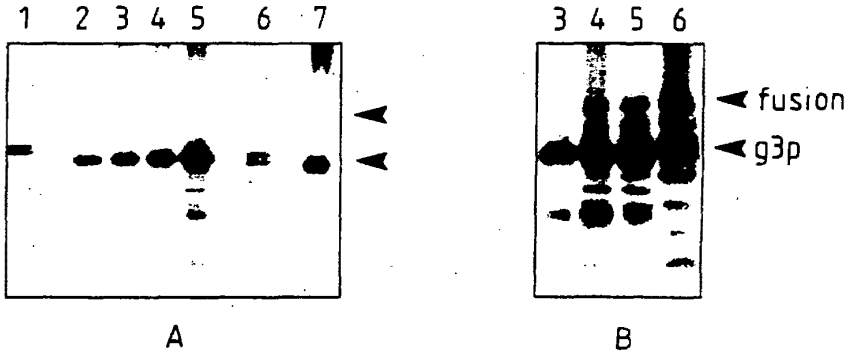


Fig. 37.

Fig.38.



35/46

Fig. 39.

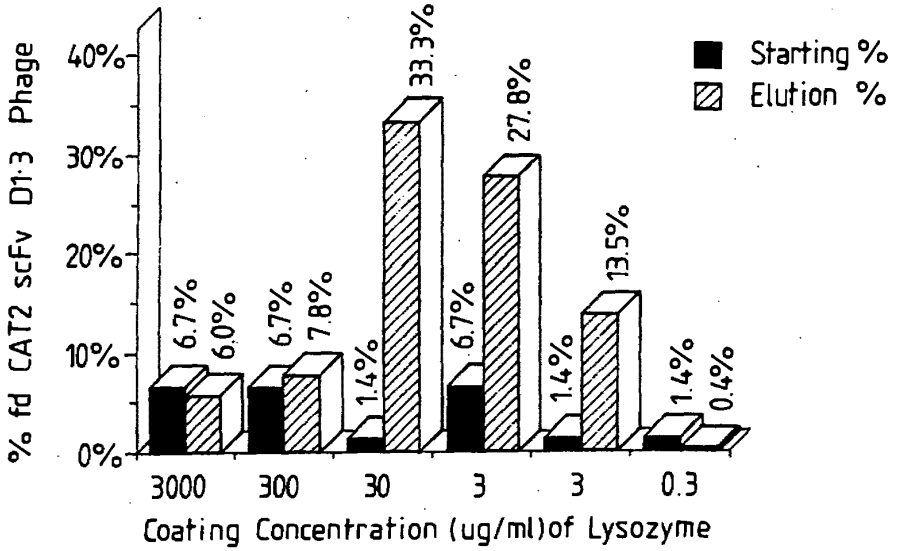
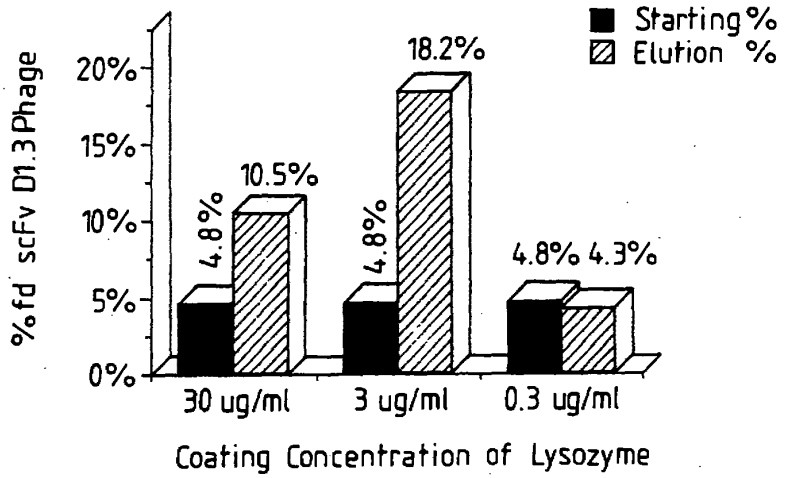


Fig. 40.



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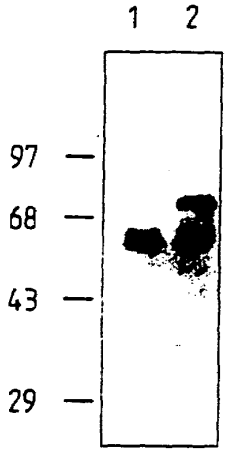

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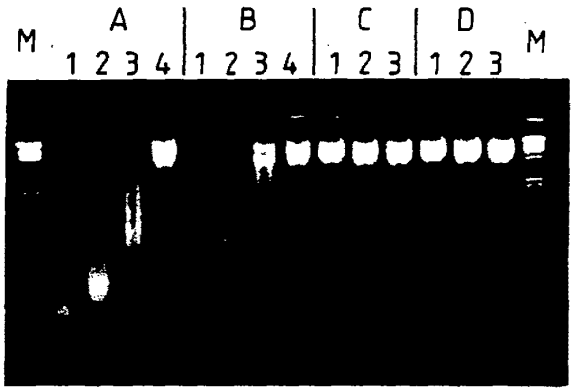

Fig.43.

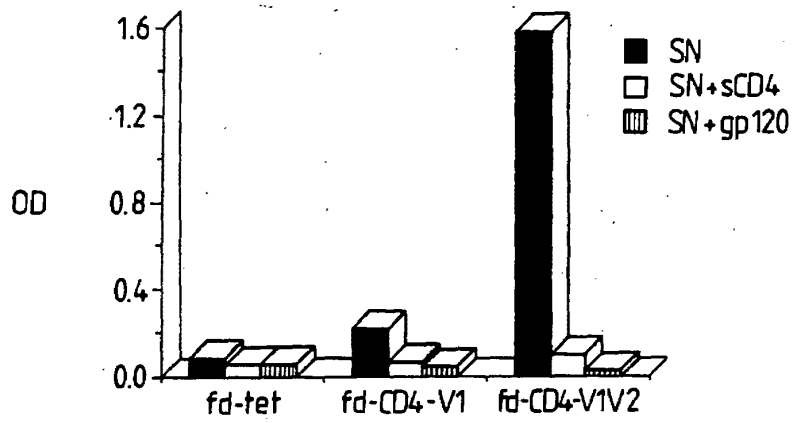

Fig. 44 (i)

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AAGATAAGAGTGTCACGTGTCCAGGTCGACGTCGTCAGACCCCGACTCGAACACTTCGGACCCCGAAGTCACTTCGACAGGACGTTCCGA
PheTyrSerHisSerAlaGlnValGlnLeuGlnGlnSerGlyAlaGluLeuValLysProGlyAlaSerValLysLeuSerCysLysAla

       100        110        120        130        140        150        160        170        180
TCTGGCTACACCTTCACCAGCTACTGGATGCACTGGGTGAAGCAGAGGCCTGGACGAGGCCTTGAGTGGATTGGAAGGATTGATCCTAAT
AGACCGATGTGGAAGTGGTCGATGACCTACGTGACCCACTTCGTCTCCGGACCTGCTCCGGAACTCACCTAACCTTCCTAACTAGGATTA
SerGlyTyrThrPheThrSerTyrTrpMetHisTrpValLysGlnArgProGlyArgGlyLeuGluTrpIleGlyArgIleAspProAsn

       190        200        210        220        230        240        250        260        270
AGTGGTGGTACTAAGTACAATGAGAAGTTCAAGAGCAAGGCCACACTGACTGTAGACAAACCCTCCAGCACAGCCTACATGCAGCTCAGC
TCACCACCATGATTCATGTTACTCTTCAAGTTCTCGTTCCGGTGTGACTGACATCTGTTTGGGAGGTCGTGTCGGATGTACGTCGAGTCG
SerGlyGlyThrLysTyrAsnGluLysPheLysSerLysAlaThrLeuThrValAspLysProSerSerThrAlaTyrMetGlnLeuSer

       280        290        300        310        320        330        340        350        360
AGCCTGACATCTGAGGACTCTGCGGTCTATTATTGTGCAAGATACGACTACGGTAGTAGCTACTACTTTGACTACTGGGGCCAAGGGACC
TCGGACTGTAGACTCCTGAGACGCCAGATAATAACACGTTCTATGCTGATGCCATCATCGATGATGAAACTGATGACCCCGGTTCCCTGG
SerLeuThrSerGluAspSerAlaValTyrTyrCysAlaArgTyrAspTyrGlySerSerTyrTyrPheAspTyrTrpGlyGlnGlyThr

       370        380        390        400        410        420        430        440        450
ACGGTCACCGTCTCCTCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCCCAGGCTGTTGGGACACAGGAATCTGCA
TGCCAGTGGCAGAGGAGTCCACCTCCGCCAAGTCCGCCTCCACCGAGACCGCCACCGCCTAGGGTCCGACAACCCTGTGTCCTTAGACGT
ThrValThrValSerSerGlyGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySerGlnAlaValGlyThrGlnGluSerAla

       460        470        480        490        500        510        520        530        540
CTCACCACATCACCTGGTGAAACAGTCACACTCACTTGTCGCTCAAGTACTGGGGCTGTTACAACTAGTAACTATGCCAACTGGGTCCAA
GAGTGGTGTAGTGGACCACTTTGTCAGTGTGAGTGAACAGCGAGTTCATGACCCCGACAATGTTGATCATTGATACGGTTGACCCAGGTT
LeuThrThrSerProGlyGluThrValThrLeuThrCysArgSerSerThrGlyAlaValThrThrSerAsnTyrAlaAsnTrpValGln

       550        560        570        580        590        600        610        620        630
GAAAAACCAGATCATTTATTCACTGGTCTAATAGGTGGTACCAACAACCGAGCTCCAGGTGTTCCTGCCAGATTCTCAGGCTCCCTGATT
CTTTTTGGTCTAGTAAATAAGTGACCAGATTATCCACCATGGTTGTTGGCTCGAGGTCCACAAGGACGGTCTAAGAGTCCGAGGGACTAA
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```

Fig. 44 (ii)

SUBSTITUTE SHEET

```
        640         650         660         670         680         690         700  C   G  710         720
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CCTCTGTTCCGACGGGAGTGGTAGTGTCCCCGTGTCTGACTCCTACTCCGTTATATAAAGACACGAGATACCATGTCGTTGGTAACCCAC
GlyAspLysAlaAlaLeuThrIleThrGlyAlaGlnThrGluAspGluAlaIleTyrPheCysAlaLeuTrpTyrLeuAsnHisTrpVal

        730         740         750         760         770
TTCGGTGGAGGAACCAAACTGACTGTCCTCGAGATCAAACGGGCGGCCGC
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Fig.45.

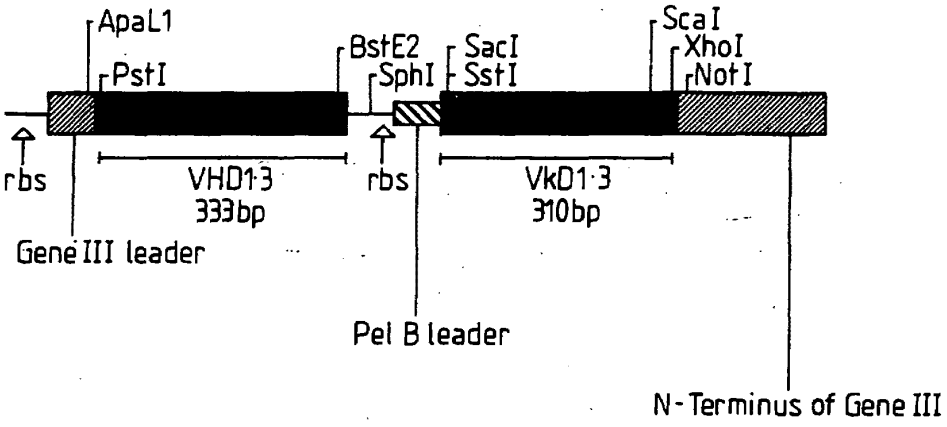

ApaL1
PstI
BstE2
SphI
SacI
SstI
ScaI
XhoI
NotI
rbs
VHD1·3
333bp
rbs
VkD1·3
310bp
Gene III leader
Pel B leader
N-Terminus of Gene III

Fig. 46.

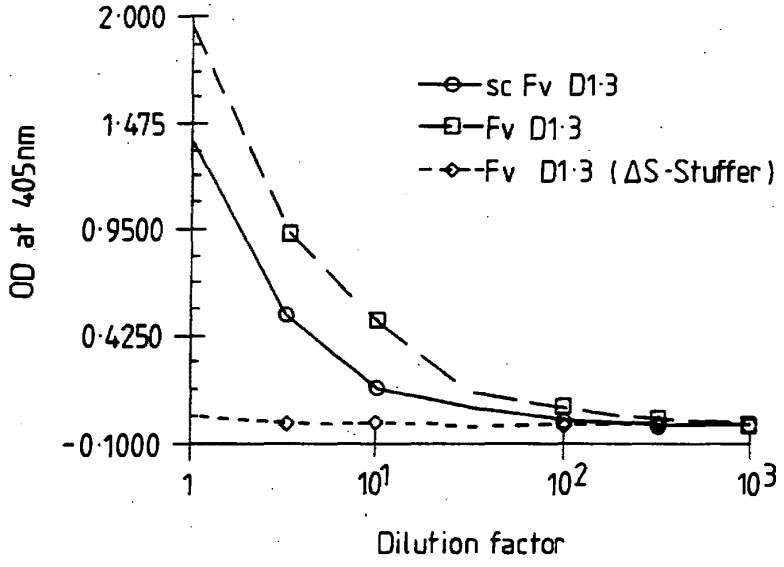
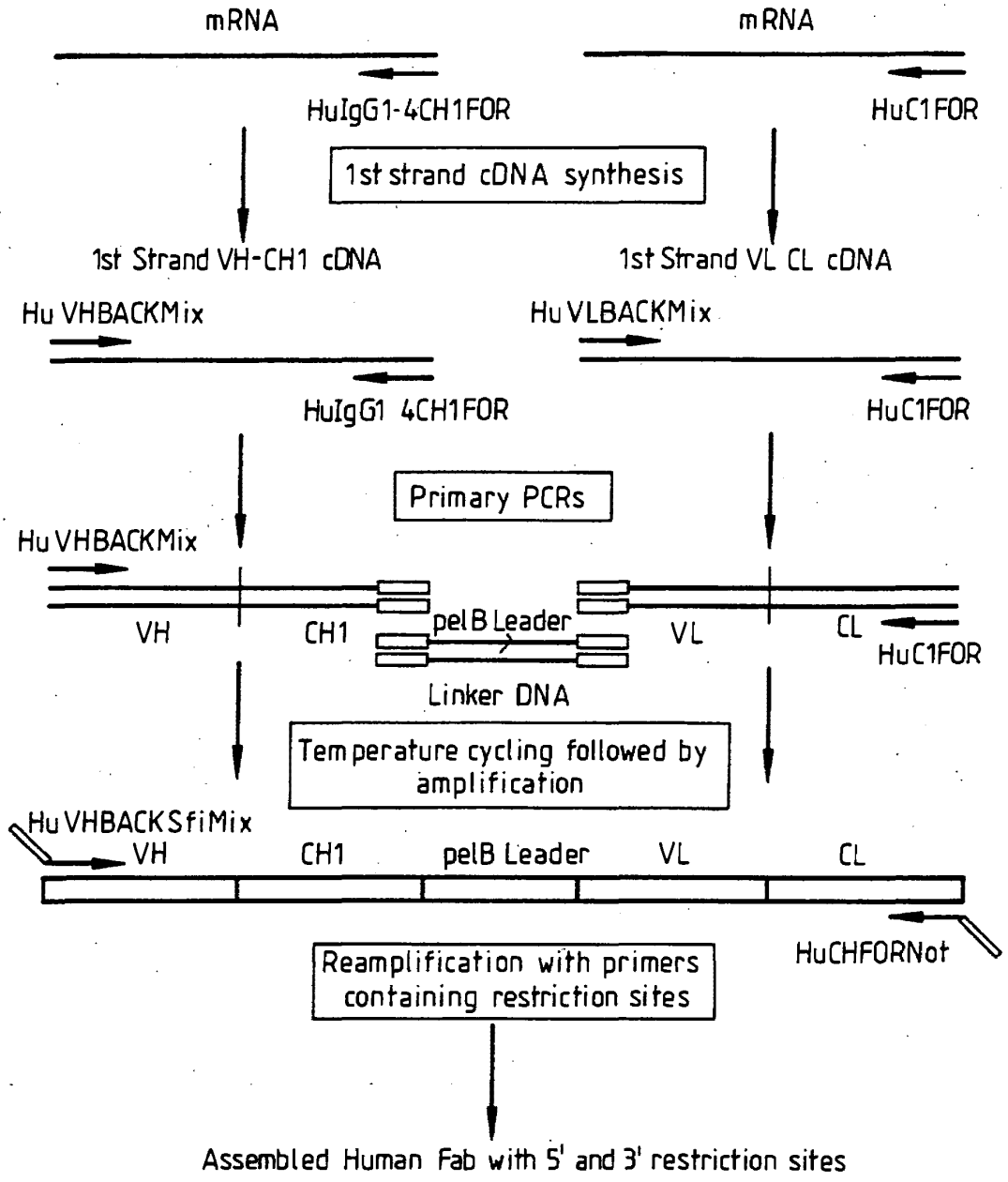

2·000
1·475
0·9500
0·4250
-0·1000
OD at 405nm
1
10¹
10²
10³
Dilution factor
sc Fv D1·3
Fv D1·3
Fv D1·3 (ΔS-Stuffer)

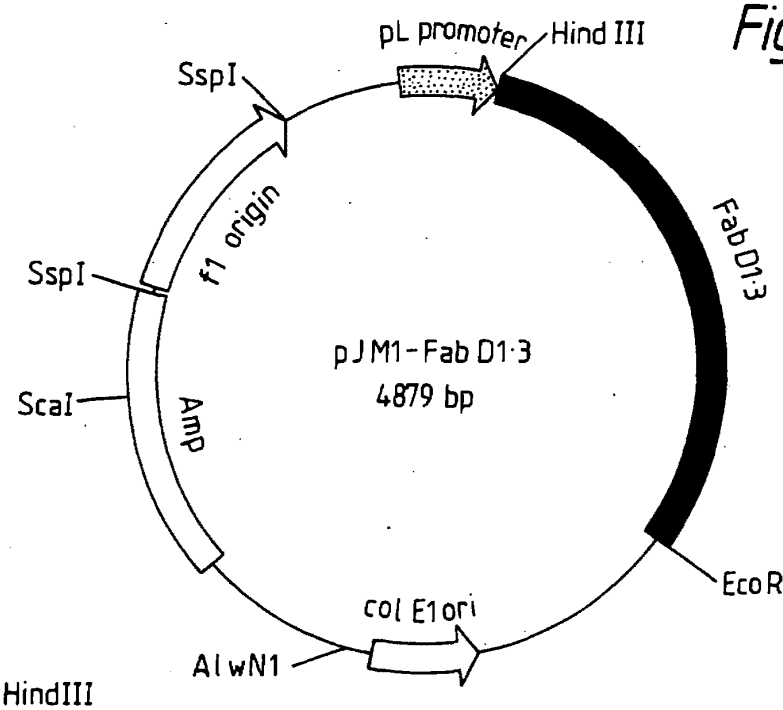
Fig. 47.



SUBSTITUTE SHEET

Fig. 48.

A.



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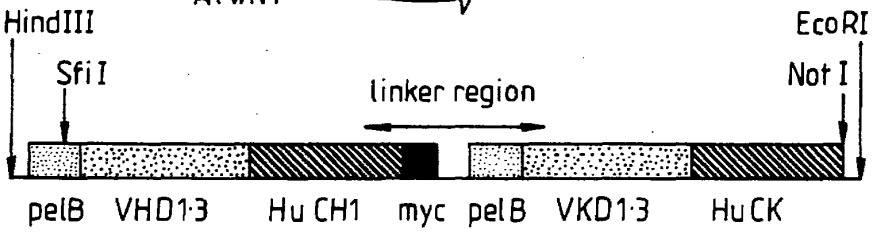


C. Sequence of linker region

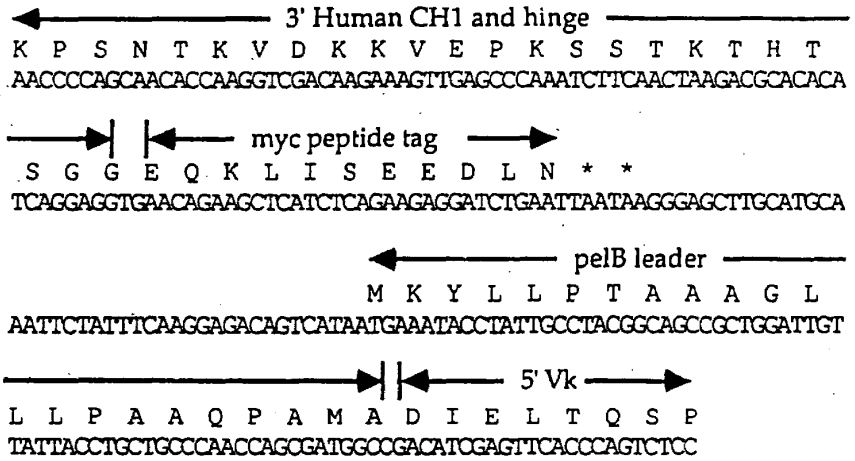


Fig. 49.

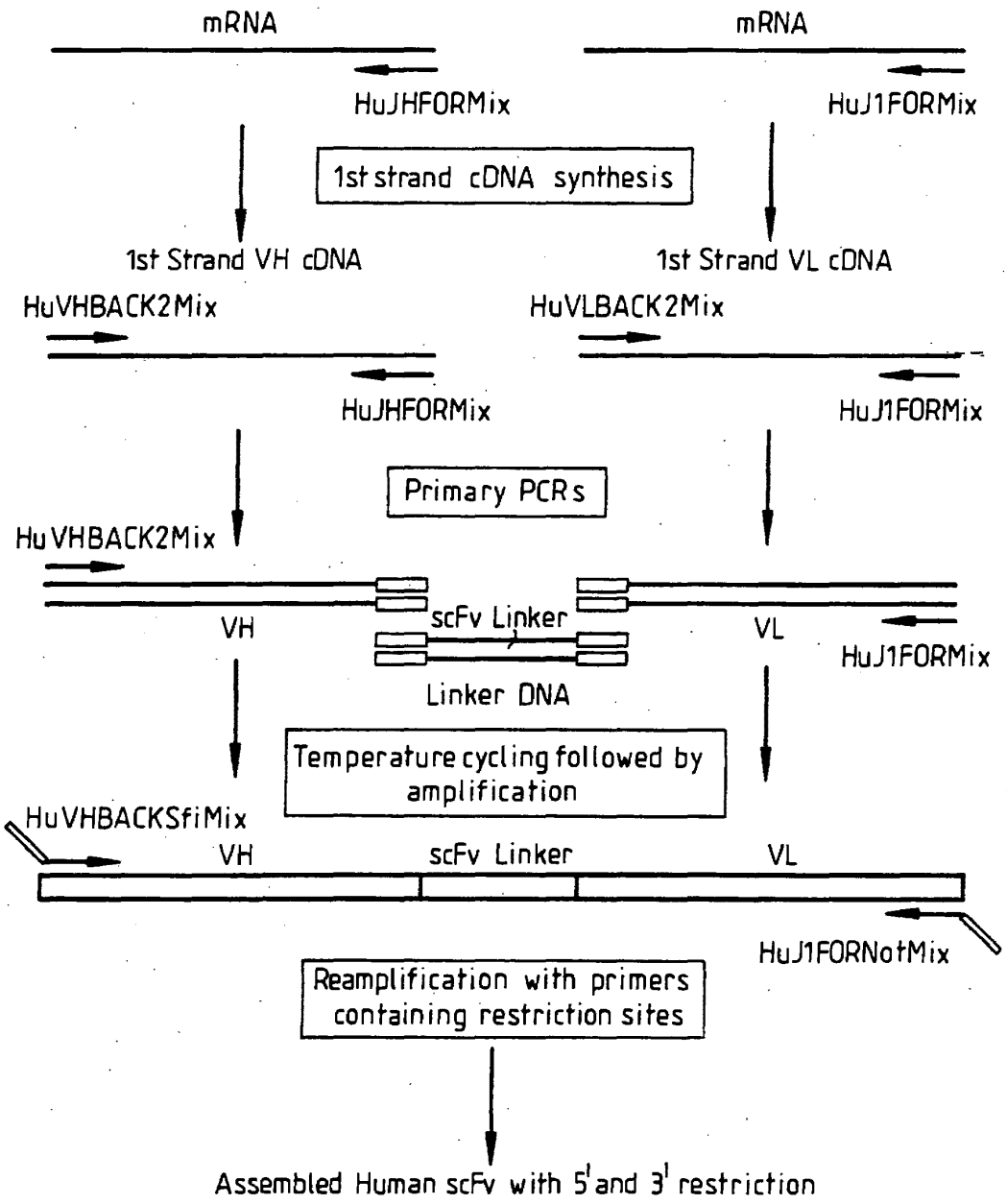
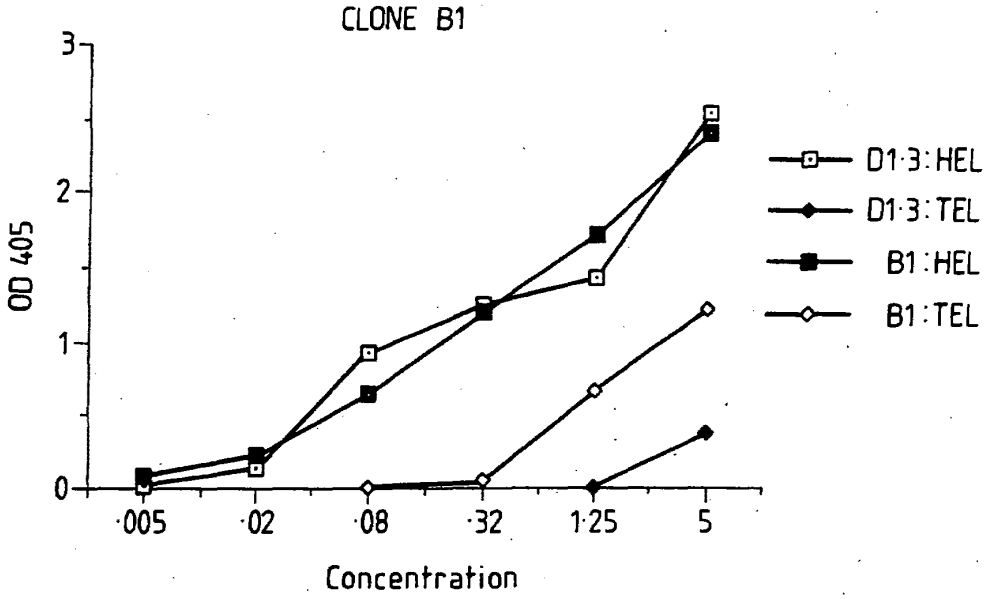


Fig. 50.



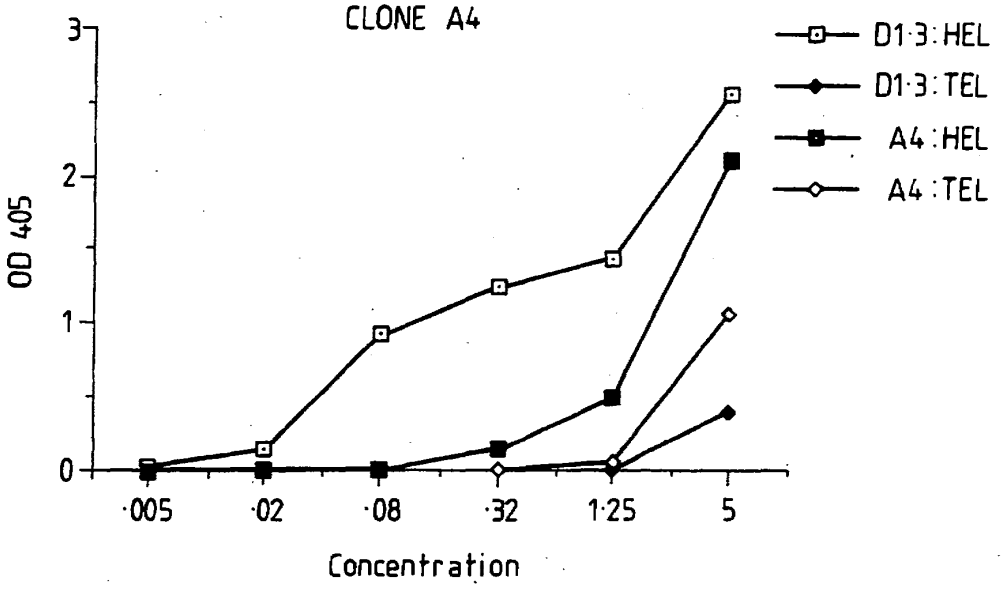


Fig.51.

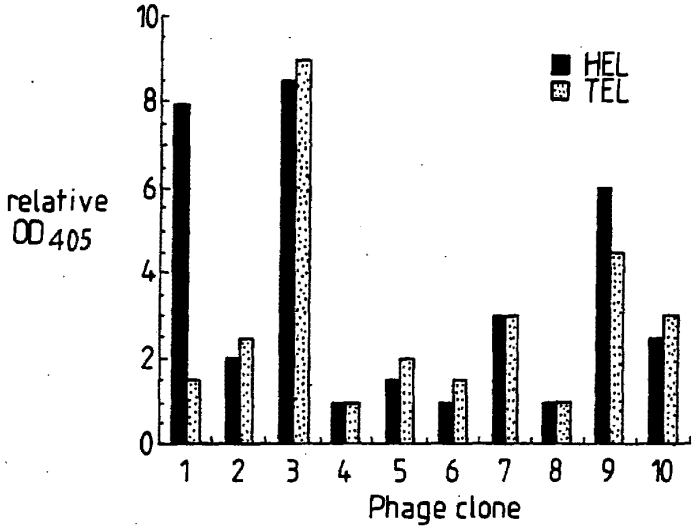


Fig.53.

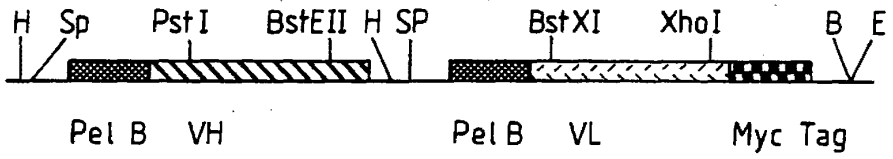


Fig. 52.

	CDR 1	CDR 2
D1.3	DIQMTQSPASLSASVGETVTITCR**ASGNIHNYLA**	WYQQKQGKSPQLLVY**YTTTLAD**
M1F	DIELTQSPSSLSASLGERVSLTCR**ASQDIGSSLN**	WLQQEPDGTIKRLIY**ATSSLDS**
M21	DIELTQSPALMAASPGEKVTITCS**VSSSISSSNLH**	WYQQKSETSPKPWIY**GTSNLAS**

	CDR 3
D1.3	GVPSRFSGSGSGTQYSLKINSLQPEDFGSYYC**QHFWSTPR**TFGGGTKLEIKR
M1F	GVPKRFSGSRSGSDYSLTISSLESEDFVDYYC**LQYASSPW**TFGGGTKLELKR
M21	GVPVRFSGSGSGTSYSLTISSMEAEDAATYYC**QQWSSYPL**TFGAGTKLEIKR

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01134

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

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II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; G01N ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	NUCLEIC ACIDS RESEARCH. vol. 16, no. 15, 1988, ARLINGTON, VIRGINIA US pages 7583 - 7600; SHORT, J.M. ET AL.: 'Lambda ZAP : a bacteriophage expression vector with in vivo excision properties.'	11-13,17
Y	See the whole document, especially Figure 1	14-16, 18-21, 24-43

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* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
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International Searching Authority	**Signature of Authorized Officer**
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	SCIENCE. vol. 246, December 8, 1989, LANCASTER, PA US pages 1275 - 1281; HUSE, W.D. ET AL.: 'Generation of large combinatorial library of the Immunoglobulin repertoire in phage Lambda '	1-4,17, 18,35-43
Y	see the whole document	5-10, 14-16, 19-21, 24-34

X	WO,A,8 806 630 (GENEX CORPORATION; USA) September 7, 1988	11-13,17
Y	see claims 1-4; figures 6,7; example 1	5-10, 14-16, 18-21, 24-43

P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, May 1991, WASHINGTON US pages 4363 - 4366; KANG, A.S.: 'Linkage of recognition and replication functions by assembling combinatorial Fab libraries along phage surface. ' see the whole document	1-43

P,X	WO,A,9 014 443 (HUSE, WILLIAM; USA) November 29, 1990 see the whole document	1-43

P,X	WO,A,9 014 424 (SCRIPPS CLINC AND RESEARCH FOUNDATION; USA) November 29, 1990 see the whole document	1-43

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9101134
SA 49532

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8806630	07-09-88	EP-A-	0349578	10-01-90
WO-A-9014443	29-11-90	AU-A-	5673390	18-12-90
		AU-A-	5813890	18-12-90
		AU-A-	5834490	18-12-90
		EP-A-	0425661	08-05-91
		WO-A-	9014424	29-11-90
		WO-A-	9014430	29-11-90
WO-A-9014424	29-11-90	AU-A-	5673390	18-12-90
		AU-A-	5813890	18-12-90
		EP-A-	0425661	08-05-91
		WO-A-	9014430	29-11-90
		AU-A-	5834490	18-12-90
		WO-A-	9014443	29-11-90

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(54) Title: SPECIFIC BINDING AGENTS

(57) Abstract

A reshaped human antibody or reshaped human antibody fragment having specificity for human polymorphic epithelial mucin (PEM) is produced by transferring the complementarity determining regions (CDRs) from a murine anti-HMFG hybridoma cell line HMFG1 into a human antibody variable region framework. The reshaped molecule can be used in the treatment or diagnosis of cancer.

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SPECIFIC BINDING AGENTS

This invention relates to specific binding agents, and in particular to polypeptides containing amino acid sequences that bind specifically to other proteinaceous or non-proteinaceous materials. The invention most particularly concerns the production of such specific binding agents by genetic engineering.

Antibody structure

Natural antibody molecules consist of two identical heavy-chain and two identical light-chain polypeptides, which are covalently linked by disulphide bonds. Figure 14 of the accompanying drawings diagramatically represents the typical structure of an antibody of the IgG class. Each of the chains is folded into several discrete domains. The N-terminal domains of all the chains are variable in sequence and therefore called the variable regions (V-regions). The V-regions of one heavy (VH) and one light chain (VL) associate to form the antigen-binding site. The module formed by the combined VH and VL domains is referred to as the Fv (variable fragment) of the

antibody. The C-terminal ends of both heavy and light chains are more conserved in sequence and therefore referred to as the constant regions. Heavy chain constant regions are composed of several domains, eg. the heavy chain constant region of the gamma-isotype (IgG) consists of three domains (CH1, CH2, CH3) and a hinge region which connects the CH1 and CH2 domains. The hinges of the two heavy chains are covalently linked together by disulphide bridges. Light chains have one constant domain which packs against the CH1 domain. The constant regions of the antibody molecule are involved in effector functions such as complement lysis and clearing by Antibody Dependant Cell Cytotoxicity (ADCC). Classical digestion of an antibody with the protease papain yields three fragments. One fragment contains the CH2 and CH3 domains and, as it crystallises easily, was called the Fc fragment. The other two fragments were designated the Fab (antigen-binding) fragments, they are identical and contain the entire light chain combined with the VH and CH1 domain. When using pepsin, the proteolytic cleavage is such that the two Fabs remain connected via the hinge and form the $(Fab)_2$ fragment. Each of the domains is represented by a separate exon at the genetic level.

The variable regions themselves each contain 3 clusters of hypervariable residues, in a framework of more conserved sequences. These hypervariable regions interact with the antigen, and are called the Complementarity Determining Regions (CDRs). The more conserved sequences are called the Framework Regions (FRs). See Kabat et al (1987). X-ray studies of antibodies have shown that the CDRs form loops which protrude from the top of the molecule, whilst the FRs provide a structural beta-sheet framework.

Modified antibodies

In one embodiment, the invention relates to so-called "reshaped" or "altered" human antibodies, ie. immunoglobulins having essentially human constant and framework regions but in which the complementarity determining regions (CDRs) correspond to those found in a non-human immunoglobulin, and also to corresponding reshaped antibody fragments.

The general principles by which such reshaped human antibodies and fragments may be produced are now well-known, and reference can be made to Jones et al (1986), Riechmann et al (1988), Verhoeyen et al (1988), and EP-A-239400 (Winter). A comprehensive list of relevant literature references is provided later in this specification.

Reshaped human antibodies and fragments have particular utility in the in-vivo diagnosis and treatment of human ailments because the essentially human proteins are less likely to induce undesirable adverse reactions when they are administered to a human patient, and the desired specificity conferred by the CDRs can be raised in a host animal, such as a mouse, from which antibodies of selected specificity can be obtained more readily. The variable region genes can be cloned from the non-human antibody, and the CDRs grafted into a human variable-region framework by genetic engineering techniques to provide the reshaped human antibody or fragment. To achieve this desirable result, it is necessary to identify and sequence at least the CDRs in the selected non-human antibody, and preferably the whole non-human variable region sequence, to allow

identification of potentially important CDR-framework interactions.

Antibodies raised against the human milk fat globule (HMFG), generally in a delipidated state, can exhibit a broad spectrum of reactivity with epithelial origin neoplasms, particularly carcinomas of the breast, ovary, uterus and lung. See Taylor-Papadimitriou et al (1981) and Arklie et al (1981). One well-characterised antibody (designated HMFG1) is known to bind to a component of the HMFG, also found in some body tissues, some cancer tissues and urine, which has been designated polymorphic epithelial mucin (PEM) (Gendler et al, 1988). Binding is thought to involve the peptide core of the PEM. Corresponding useful specificity can be achieved by raising antibodies against cancer cells, for example breast cancer cell lines.

EP-A2-0369816 (The University of Melbourne, Xing et al) describes monoclonal antibodies specific for human polymorphic epithelial mucin, which bind to a defined amino acid sequence. It is suggested in EP-A2-0369816 that the described antibodies may be "humanised" according to the method of Riechmann et al (1988). However, Xing et al do not describe the actual preparation of any such reshaped anti-PEM antibodies.

Summary of the invention

The invention provides, as one embodiment, a synthetic specific binding polypeptide having specificity for a polymorphic epithelial mucin (PEM), and especially a synthetic specific binding polypeptide having anti-human milk fat globule (HMFG) specificity, containing one or more of the CDRs depicted in Figures 1 and 2 of the

accompanying drawings. By synthetic, we particularly mean that the polypeptide is produced by recombinant DNA technology, and to that extent at least is different from a naturally-occurring or naturally-induced specific binding agent having identical specificity. Alternatively, the synthetic polypeptide has been produced by artificially assembling a sequence of amino acids to produce a novel or nature-identical molecule. The synthetic polypeptide can be equivalent to an intact conventional antibody, or equivalent to a multiple or single-chain fragment of such an antibody, or can be simply a material that includes one or more sequences that confer the desired specific binding capability.

The invention provides as an important embodiment a reshaped human antibody, or a reshaped human antibody fragment, having anti-PEM specificity, and especially having anti-HMFG specificity, containing one or more of the CDRs depicted in Figures 1 and 2 of the accompanying drawings. Preferably, the reshaped antibody or fragment of the invention contains all 3 of the CDRs depicted in Figure 1 of the accompanying drawings, in a human heavy chain variable region framework. Alternatively, or in addition, the reshaped antibody or fragment of the invention contains all 3 of the CDRs depicted in Figure 2 of the accompanying drawings, in a human light chain variable region framework.

Another embodiment of the invention is a reshaped antibody or reshaped antibody fragment containing a protein sequence as depicted in Figure 12 and/or Figure 13 of the accompanying drawings.

Other important embodiments of the invention are an expression vector incorporating a DNA sequence as depicted

in Figure 12 and/or Figure 13 of the accompanying drawings, and an expression vector incorporating a DNA sequence encoding one or more of the protein sequences designated as being a CDR in Figure 1 and/or Figure 2 of the accompanying drawings.

An important aspect of the invention is a stable host cell line containing a foreign gene that causes the host cell line to produce a specific binding agent according to the invention. This can be a stable host cell line containing a foreign gene that encodes at least one of the amino acid sequences designated as being a CDR in Figure 1 and/or Figure 2 of the accompanying drawings, together with a protein framework that enables the encoded amino acid sequence when expressed to function as a CDR having specificity for HMFG.

The invention also provides an immortalised mammalian cell line, or a yeast, or other eukaryotic cell, or a prokaryotic cell such as a bacterium, producing a reshaped antibody or fragment according to the invention.

Another important aspect of the invention is a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, having specificity equivalent to that of the gamma-1, kappa anti-HMFG monoclonal antibody "HMFG1".

The invention also provides two novel plasmids, pSVgpt-HuVHHMFG1-HuIgG1 and pSVneo-HuVkHMFG1-HuCk, and these plasmids can be used in the production of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

These plasmids are contained in novel E.coli strains NCTC 12411 and NCTC 12412, respectively.

Other aspects of the invention are:

a) A DNA sequence encoding a reshaped human antibody heavy-chain variable region having specificity for HMFG, as contained in E.coli NCTC 12411.

b) A DNA sequence encoding a reshaped human antibody light-chain variable region having specificity for HMFG, as contained in E.coli NCTC 12412.

c) A reshaped human antibody heavy-chain variable region having specificity for HMFG, producible by means of the expression vector contained in E.coli NCTC 12411.

d) A reshaped human antibody light-chain variable region having specificity for HMFG, producible by means of the expression vector contained in E.coli NCTC 12412.

e) A reshaped human antibody or reshaped human antibody fragment, comprising at least one variable region according to c) or d) above.

A particular embodiment of the invention is therefore a reshaped human antibody or reshaped human antibody fragment possessing anti-HMFG specificity and incorporating a combination of CDRs (which may, for example, be cloned from a murine anti-HMFG immunoglobulin) having the amino acid sequences identified as CDR1, CDR2 and CDR3 respectively in Figures 1 and 2 of the accompanying drawings, which respectively represent the heavy chain variable region (VH) and light chain variable region (Vk) of a murine anti-HMFG monoclonal antibody that

we have cloned and sequenced. In the case of an intact antibody, or a fragment comprising at least one heavy chain variable region and at least one light chain variable region, the reshaped antibody or fragment preferably contains all six CDRs from the non-human source. To be most effective in binding, the CDRs should preferably be sited relative to one another in the same arrangement as occurs in the original non-human antibody, e.g. the VH CDRs should be in a human VH framework, and in the order in which they occur naturally in the non-human antibody.

As will be apparent to those skilled in the art, the CDR sequences and the surrounding framework sequences can be subject to modifications and variations without the essential specific binding capability being significantly reduced. Such modifications and variations can be present either at the genetic level or in the amino acid sequence, or both. Accordingly, the invention encompasses synthetic (reshaped) antibodies and fragments that are functionally equivalent to those described herein having precisely defined genetic or amino acid sequences.

The invention can also be applied in the production of bi-specific antibodies, having two Fab portions of different specificity, wherein one of the specificities is conferred by a reshaped human variable chain region incorporating one or more of the CDRs depicted in Figures 1 and 2 of the accompanying drawings.

The invention can also be applied in the production of so-called single-chain antibodies (for example, as disclosed in Genex EP-A-281604), and also to polysaccharide-linked antibodies (see Hybritech EP-A-315456), and other modified antibodies.

Any human constant regions (for example, gamma 1, 2, 3 or 4-type) can be used.

Antibody fragments retaining useful specific binding properties can be $(Fab)_2$, Fab, Fv, VH or Vk fragments. These can be derived from an intact reshaped antibody, for example by protease digestion, or produced as such by genetic engineering.

Practical applications of the invention

An important aspect of the invention is a reshaped human anti-HMFG antibody or fragment, as defined above, linked to or incorporating an agent capable of retarding or terminating the growth of cancerous cells, or to an imaging agent capable of being detected while inside the human body. The invention also includes injectable compositions comprising either of such combinations in a pharmaceutically acceptable carrier, such as saline solution, plasma extender or liposomes. The invention also includes the use, in a method of human cancer therapy or imaging, of a reshaped human anti-HMFG antibody or fragment as defined above. The invention further includes the use of such an antibody or fragment for the manufacture of a medicament for therapeutic application in the relief of cancer in humans, or the use of such an antibody or fragment in the manufacture of a diagnostic composition for in-vivo diagnostic application in humans.

The Fc region of the antibody, itself using pathways and mechanisms available in the body, such as complement lysis and antibody dependent cellular cytotoxicity, can be used to affect adversely the growth of cancerous cells. In this embodiment, no additional reagent need be linked to the reshaped antibody.

Examples of agents capable of affecting adversely the growth of cancerous cells include radioisotopes, such as Yttrium 90 and Iodine 131; drugs such as methotrexate; toxins such as ricin or parts thereof; and enzymes which may for example turn an inactive drug into an active drug at the site of antibody binding.

Examples of imaging agents include radioisotopes generating gamma rays, such as Indium 111 and Technetium 99; radioisotopes generating positrons, such as Copper 64; and passive agents such as Barium which act as contrast agents for X-rays, and Gadolinium in nmr/esr scanning.

In order to link a metallic agent, such as a radioisotope, to a specific binding agent of the invention, it may be necessary to employ a coupling or chelating agent. Many suitable chelating agents have been developed, and reference can be made for example to US 4824986, US 4831175, US 4923985 and US 4622420. Techniques involving the use of chelating agents are described, for example, in US 4454106, US 4722892, Moi et al (1988), McCall et al (1990), Deshpande et al (1990) and Meares et al (1990).

The use of radiolabelled antibodies and fragments in cancer imaging and therapy in humans is described for example in EP 35265. It may be advantageous to use the radiolabelled cancer-specific antibody or fragment in conjunction with a non-specific agent radiolabelled with a different isotope, to provide a contrasting background for so-called subtraction imaging.

The antibody reagents of the invention can be used to identify, e.g. by serum testing or imaging, and/or to treat, PEM-producing cancers. Such cancers can occur as

for example, carcinomas of breast, ovary, uterus and lung, or can manifest themselves as liquids such as pleural effusions.

Modified antibody production

The portions of the VH and VL regions that by convention (Kabat, 1987) are designated as being the CDRs may not be the sole features that need to be transferred from the non-human monoclonal antibody. Sometimes, enhanced antibody performance, in terms of specificity and/or affinity, can be obtained in the reshaped human antibody if certain non-human framework sequences are conserved in the reshaped human antibody. The objective is to conserve the important three-dimensional protein structure associated with the CDRs, which is supported by contacts with framework residues.

The normal starting point from which a reshaped antibody in accordance with the invention can be prepared, is a cell (preferably an immortalised cell line), derived from a non-human host animal (for example, a mouse), which expresses an antibody having specificity against HMFG or PEM. Such a cell line can, for example, be a hybridoma cell line prepared by conventional monoclonal antibody technology. Preferably, the expressed antibody has a high affinity and high specificity for HMFG, because it should be anticipated that some loss of affinity and/or specificity may occur during the transfer of these properties to a human antibody or fragment by the procedures of the invention. By selecting a high specificity antibody as the parent antibody, the likelihood that the final reshaped antibody or fragment will also exhibit effective binding properties is enhanced.

The next stage is the cloning of the cDNA from the cell expressing the selected non-human antibody, and sequencing and identification of the variable region genes including the sequences encoding the CDRs. The experimental procedures involved can now be regarded as routine in the art, although they are still laborious.

If the object is to produce a reshaped complete human antibody, or at least a fragment of such an antibody which will contain both heavy and light variable domains, it will be necessary to sequence the cDNA associated with both of these domains.

Once the relevant cDNA sequence or sequences have been analysed, it is necessary to prepare one or more replicable expression vectors containing a DNA sequence which encodes at least a variable domain of an antibody, which variable domain comprises human framework regions together with one or more CDRs derived from the selected non-human anti-HMFG antibody. The DNA sequence in each vector should include appropriate regulatory sequences necessary to ensure efficient transcription and translation of the gene, particularly a promoter and leader sequence operably linked to the variable domain sequence. In a typical procedure to produce a reshaped antibody or fragment in accordance with the invention, it may be necessary to produce two such expression vectors, one containing a DNA sequence for a reshaped human light chain and the other, a DNA sequence for a reshaped human heavy chain. The expression vectors should be capable of transforming a chosen cell line in which the production of the reshaped antibody or fragment will occur. Such a cell line may be for example, a stable non-producing myeloma cell line, examples (such as NS0 and sp2-0) of which are readily available commercially. An alternative is to use

a bacterial system, such as E.coli, as the expression vehicle for the reshaped antibody or fragment. The final stages of the procedure therefore involve transforming the chosen cell line or organism using the expression vector or vectors, and thereafter culturing the transformed cell line or organism to yield the reshaped human antibody or fragment.

By way of example only, detailed steps by means of which appropriate expression vectors can be prepared are given later in this specification. The manipulation of DNA material in a suitably equipped laboratory is now a well-developed art, and the procedures required are well within the skill of those versed in this art. Many appropriate genomic and cDNA libraries, plasmids, restriction enzymes, and the various reagents and media which are required in order to perform such manipulations, are available commercially from suppliers of laboratory materials. For example, genomic and cDNA libraries can be purchased from Clontech Laboratories Inc. The steps given by way of example below are purely for the guidance of the reader of this specification, and the invention is in no way critically dependant upon the availability of one or more special starting materials. In practice, the skilled person has a wide range of materials from which to choose, and can exploit and adapt the published technology using acquired experience and materials that are most readily available in the scientific environment. For example, many plasmids fall into this category, having been so widely used and circulated within the relevant scientific community that they can now be regarded as common-place materials.

Examples

The procedure used to prepare reshaped anti-HMFG human antibodies is described in detail below, by way of example only, with reference to the accompanying drawings, of which:

Figure 1 shows the cDNA sequence coding for a murine heavy chain variable region having anti-HMFG specificity. The 3 classical CDRs are indicated, together with an amino acid sequence matching the cDNA code.

Figure 2 shows the cDNA sequence coding for a murine light chain variable region having anti-HMFG specificity.

Figure 3a shows a design for a synthesic reshaped human VH gene with HMFG1 specificity (HuVHIconHMFG1 gene cassette) containing 3 fragments.

Figures 3b to 3d show the sequence of the respective fragments in Figure 3a, and also the oligonucleotides used in the assembly of each fragment.

Figures 4a, 4b and 4c together show a route by which an expression vector encoding a reshaped human heavy chain incorporating the CDRs of Figure 1, can be prepared.

Figures 5a and 5b together show a similar transformation route to obtain an expression vector encoding a reshaped human light chain incorporating the CDRs of Figure 2, can be prepared.

Figure 6 shows the plasmid pUC12-IgEnh, which contains an enhancer sequence used in the routes of Figures 4a to 5b.

Figure 7 shows the source of plasmid pBGS18-HulgG1 used in the route of Figure 4c.

Figure 8 shows the source of plasmid pBGS18-HuCk used in the route of Figure 5b.

Figure 9 shows two synthetic oligonucleotide sequences I and II used in cloning the cDNA sequences of Figures 1 and 2.

Figure 10 shows two synthetic oligonucleotide sequences III and IV used to introduce the Kpn I and Sal I restriction sites in M13mp9HuVHLYS respectively, in the route depicted in Figure 4a.

Figure 11 shows three synthetic oligonucleotide sequences VI, VII and VIII used to graft the Vk HMFG1 CDRs onto the human VK REI framework regions in the route depicted in Figure 5a.

Figures 12 and 13 show the cDNA and amino acid sequences of the resulting reshaped human heavy and light chain variable regions respectively.

Figure 14 depicts in diagramatic form the structure of a typical antibody (immunoglobulin) molecule.

Figure 15 shows in graphical form the relative specific anti-HMFG1 binding activity of the resulting reshaped human antibody.

The experimental procedures required to practice the invention do not in themselves represent unusual technology. The cloning and mutagenesis techniques were performed as generally described for example in Verhoeyen

et al (1988); Riechmann et al (1988) and EP-A-239400 (Winter). The "de novo" synthesis of a reshaped human heavy chain variable region gene (see Figures 3a - 3d) was done by conventional techniques, using a set of long overlapping oligonucleotides (see also Jones et al, 1988). Laboratory equipment and reagents for synthesising long oligonucleotides are readily available, and as techniques in this field develop it is becoming practicable to synthesise progressively longer sequences.

Detailed laboratory manuals, covering all basic aspects of recombinant DNA techniques, are available, e.g. "Molecular Cloning" by Sambrook et al (1989).

By means of the invention, the antigen binding regions of a mouse anti-HMFG antibody (HMFG1) were grafted onto human framework regions. The resulting reshaped human antibody (designated HuHMFG1) has binding characteristics similar to those of the original mouse antibody.

Such reshaped antibodies can be used for in vivo diagnosis and treatment of human cancers, eg. ovarian cancers and breast cancers, and are expected at least to reduce the problem of an immune response in the patient often seen upon administration of non-human antibody. A similar benefit has been shown for reshaped CAMPATH-1 antibody in Hale et al (1988).

Methods:

1. Cloning and sequence determination of the mouse variable region genes

Messenger RNA was isolated from a murine hybridoma line which secretes the gamma-1, kappa anti-HMFG antibody "HMFG1" (see Taylor-Papadimitriou et al, 1981 and Arklie et al, 1981). First strand cDNA was synthesised by priming with oligonucleotides I and II (see Figure 9) complementary to the 5' ends of the CH1 and Ck exons respectively. Second strand cDNA was obtained as described by Gübler and Hoffmann (1983).

Kinased EcoRI linkers were ligated to the heavy chain double-stranded cDNA and Pst1 linkers to the light chain double-stranded cDNA (both were first treated with EcoRI or PstI methylase to protect possible internal sites), followed by cloning into EcoRI or PstI-cut pUC9 (Vieira et al, 1982) and transformation of E.coli strain TG2 (Gibson, 1984).

Colonies containing genes coding for murine HMFG1 VH (MoVHHMFG1) and for murine anti-HMFG Vk (MoVkHMFG1) were identified by colony hybridisation with 2 probes consisting respectively of 32P-labelled first strand cDNA of HMFG1 VH and Vk. Positive clones were characterised by plasmid preparation, followed by EcoRI or PstI digestion and 1.5% agarose gel analysis. Full-size inserts (about 450bp) were subcloned in the EcoRI or PstI site of M13mp18 (Norrander et al, 1983). This yielded clones with inserts in both orientations, facilitating nucleotide sequence determination of the entire insert, by the dideoxy chain termination method (Sanger et al, 1977).

The nucleotide sequences, and their translation into amino acid sequences, of the mature variable region genes MoVHHMFG1 and MoVkHMFG1, are shown in Figures 1 and 2. The 450 bp inserts included a signal sequence and 5' untranslated sequences and linkers, not shown in the Figures.

2. Grafting of the mouse HMFG1 CDRs onto human framework regions

The general techniques necessary to achieve this have been described very adequately in Jones et al (1986), Verhoeyen et al (1988), Riechmann et al (1988) and in EP-A-239400 (Winter).

a) Light chain:

The basic construct used for reshaping a human light chain was M13mp9HuVkLYS (Riechmann et al, 1988), which contains framework regions with sequences based on those of the light chain variable regions of the human Bence-Jones protein REI (Epp et al, 1974).

The CDRs in this construct (Figure 5a) were replaced by site-directed mutagenesis with oligonucleotides VI, VII and VIII encoding the HMFG1 kappa chain CDRs flanked by 12 nucleotides at each end encoding the corresponding human framework residues. These oligonucleotides are shown in Figure 11. The mutagenesis was done as described in Riechmann et al (1988). The resulting reshaped human light chain variable region gene (HuVkHMFG1) is shown in Figure 13.

b) Heavy chain:

A reshaped human heavy chain variable region gene was obtained by "de novo" synthesis. In the experiments published by Jones et al, etc, mentioned above, rodent heavy chain CDRs were grafted onto the framework regions of the human NEW heavy chain variable region. It was shown by Verhoeyen et al (1988) and by Riechmann et al (1988) that it is important that the human framework can support the rodent CDRs in a conformation similar to the one occurring in the original rodent antibody, and that certain CDR-framework interactions can be critical. It follows thus that the more dissimilar the rodent and the human framework sequences are, the less the chance will be for the CDR graft to "take".

Comparison of the heavy chain variable region amino acid sequence of the mouse HMFG1 (Figure 1) to that of the human NEW (as used in Verhoeyen et al, 1988), revealed 44% differences between their respective framework regions. A much better homology was found when comparing to human heavy chain variable regions of subgroup I (Kabat et al, 1987); human VHNEW belongs to subgroup II.

We therefore decided to synthesise a human heavy chain variable region gene of subgroup I, containing the HMFG1 heavy chain CDRs. We designed a consensus sequence for human heavy chain subgroup I variable regions, based on sequence information on this subgroup in Kabat et al, 1987. Optimal codon usage was taken from the sequences of mouse constant region genes (the genes are expressed in a mouse myeloma line).

There are only 14% differences between the framework sequences of the HMFG1 VH and the VH of this human VH subgroup I consensus sequence (HuVHIcon). The resulting reshaped gene was designated the name HuVHIconHMFG1, and is depicted in Figure 12. The gene synthesis is described separately in section (c) below. The newly synthesised gene HuVHIconHMFG1 was used to replace HuVHLYS in the construct M13mp9HuVHLYS (Verhoeyen et al, 1988), yielding the vector M13mp9HuVHIconHMFG1 (see Figure 4a).

3. Assembly of reshaped human antibody genes in expression vectors

The next stage involved the use of a murine heavy chain enhancer IgEnh, described in Neuberger et al (1983) where the enhancer is contained in a 1kb Xbal fragment of plasmid pSV-Vμ1. The 700bp Xbal/EcoRI subfragment of this 1kb Xbal fragment is sufficient to confer enhancer activity.

An alternative source of this enhancer is plasmid pSVneoHuVkPLAP (see Fig. 5a), a variation of which has been deposited in an E.coli strain under the Budapest Treaty on 19 April 1990 as NCTC 12390. As deposited, the plasmid also contains a human kappa-chain constant region gene (cloned in the BamH1 site).

The reshaped human genes as prepared in sections 2(a) and 2(b) above, were excised from the M13 vectors as HindIII - BamHI fragments. The heavy chain variable region genes were cloned into a vector based on pSV2gpt (Mulligan et al, 1981) and the light chain variable region genes cloned into a vector based on pSV2neo (Southern et al, 1981) expression vectors, both containing the immunoglobulin heavy chain enhancer IgEnh. In the pSV2gpt

based antibody expression vector (see Fig. 4b - 4c), the Xbal/EcoRI enhancer containing fragment was cloned in the unique EcoRI site of the pSV2gpt vector (after ligating EcoRI linkers to the filled in Xbal end of the fragment).

In the pSVneo based antibody expression vector (see Fig. 5a - 5b), the 1kb Xbal enhancer containing fragment was first cloned into pUC12 (Vieira et al, 1982), yielding the plasmid pUC12-IgEnh, see Figure 6. The enhancer can then be cut out as a 700bp EcoRI/HindIII fragment (either orientation of the enhancer will work). This 700bp EcoRI/HindIII fragment is present in the plasmid pSVneoHuVkPLAP, that we used to clone the HuVkHMFG1-containing fragment described in section 2a, see Fig. 5a and 5b. The HindIII site in the original pSV2neo had been removed. It is possible to use pSV2gpt as an alternative vector for light chain expression, as in practice there is no need for neo selection.

The HuVHIconMFG1 gene was linked to a human gamma 1 constant region (Takahashi et al, 1982), cloned initially as a 8kb HindIII fragment into the HindIII site of pBGS18 (Spratt et al, 1986), and then in the pSV2gpt expression vector as a BamHI fragment (see Figures 4c and 7). It should be noted that in the Takahashi et al (1982) reference there is an error in Figure 1: the last (3') two sites are BamH1 followed by HindIII, and not the converse. This was confirmed by Flanagan et al (1982).

The HuVkHMFG1 gene was linked to a human C kappa constant region (Hieter et al, 1980) also cloned in as a BamHI fragment (see Figures 5b and 8). The source of the human Ck used in Figure 8 is given in Hieter et al (1980). The 12 kb BamH1 fragment from embryonic DNA (cloned in a

gamma Ch28 vector system) was subcloned in the BamH1 site of plasmid pBR322.

4. "de novo" synthesis of the HuVHIconHMFG1 gene

We decided to synthesise a gene encoding a human variable region gene of subgroup I (Kabat et al, 1987), and with the CDRs of VHHMFG1 (Figure 1). In summary, the synthetic gene is designed in such a way that it can substitute the HuVHLYS gene in the existing M13mp9HuVHLYS vector. The M13mp9HuVHLYS was mutagenized to contain a KpnI and SalI site at the appropriate places (see also Figure 4a), to enable cloning of the newly synthesized gene as a KpnI-SalI fragment.

The gene sequence was designed as described above in section 2(b) and is depicted in Figure 12. To facilitate the substitution of this gene for the HuVHLYS gene in M13mp9HuVHLYS (Verhoeyen et al, 1988, see also Figure 4a), 5' and 3' extensions were added to the gene. The 5' extension contains 37 bp of the leader intron and 11 bp of the second half of the leader exon (as in M13mp9HuVHLYS), and has a KpnI site at the very 5' end. The 3' extension contains 38 untranslated nucleotides (as in M13mp9HuVHLYS) and ends in a SalI site.

M13mp9HuVHLYS was modified by site directed mutagenesis with oligonucleotides III and IV to contain a KpnI and SalI site at the appropriate places (see Figure 4a and Figure 10). This vector was named M13mp9HuVHLYS(K,S). This enabled cloning of the HuVHIconHMFG1 gene as a KpnI-SalI fragment in KpnI-SalI cut M13mp9HuVHLYS(K,S) vector.

For practical reasons it was decided to synthesise the gene as three fragments (cassettes), which were then assembled in one complete gene.

Each fragment contains one of the three VHHMFG1 CDRs, and can easily be cloned or removed by using the (existing or newly introduced) unique restriction sites (see Figure 3a). Each fragment was elongated at the 5' and 3' end to create a HindIII and BamHI site respectively, to enable cloning in pEMBL9 (Dente et al, 1983). The coding strand of each fragment was divided in oligonucleotides with an average length of 33 bases. The same was done for the non-coding strand, in such a way that the oligonucleotides overlapped approximately 50% with those of the coding strand.

The sequences of each fragment and of the oligonucleotides used for assembly, are shown in Figures 3b, 3c and 3d.

Before assembling the fragments, the 5' ends of the synthetic oligonucleotides had to be phosphorylated in order to facilitate ligation. Phosphorylation was performed as follows: equimolar amounts (50 pmol) of the oligonucleotides were pooled and kinased in 40 µl reaction buffer with 8 units polynucleotide kinase for 30-45 minutes at 37°C. The reaction was stopped by heating for 5 minutes at 70°C and ethanol precipitation. Annealing was done by dissolving the pellet in 30 µl of a buffer containing: 7 mM TrisCl pH 7.5, 10 mM 2-mercapto-ethanol, 5 mM ATP were added. Subsequently the mixture was placed in a waterbath at 65°C for 5 minutes, followed by cooling to 30°C over a period of 1 hour. MgCl2 was added to a final concentration of 10 mM. T4 DNA-ligase (2.5 units) was added and the mixture was placed at 37°C for 30 min.

(or overnight at 16°C). After this the reaction mixture was heated for 10 minutes at 70°C. After ethanol precipitation the pellet was dissolved in digestion buffer and cut with HindIII and BamHI. The mixture was separated on a 2% agarose gel and the fragment with a length corresponding to the correctly assembled cassette was isolated by electro-elution.

The fragments (1, 2, 3) were ligated in pEMBL9 (cut with HindIII/BamHI), yielding the vectors pUR4107, pUR4108 and pUR4109 respectively. The sequence of the inserts was checked by sequence analysis (in both orientations). Fragment 1 was isolated from pUR4107 by KpnI/XhoI digestion, whilst fragment 2 was isolated from pUR4108 by XhoI/SacI digestion, after which they were ligated in KpnI/SacI cut pUR4109 in a three-fragment ligation. The resulting plasmid was named pUR4110 (see Figure 4a). Sequencing analysis showed that the insert contained the desired HuVHIconHMFG1 gene. This gene was cloned in a pSV2gpt-derived expression vector as depicted in Figures 4b and 4c. The vector pSVgptMoVHLYS-MoIgG1 (Verhoeyen et al, 1988) was used as the source of a pSVgpt-based vector containing the IgEnh enhancer.

5. Expression in myeloma cells

Co-transfection of the expression plasmids pSVgptHuVHIconHMFG1-HuIgG1 and pSVneoHuVkHMFG1-HuCk (Figures 4c and 5b) into NSO myeloma cells was done by electroporation (Potter et al, 1984), after linearisation with PvuI. Transfectomas were selected in mycophenolic acid containing medium to select for cells expressing the gpt gene product, and screened for antibody production and anti-HMFG activity by ELISA assays.

Clones positive for both assays were obtained and subcloned by limiting dilution and pure clones were assayed again for anti-HMFG activity, and the best producing clones were grown in serum-free medium for antibody production.

6. Deposited plasmids

E.coli strains containing plasmids used in the above procedure have been deposited, in accordance with the provisions of the Budapest Treaty, in the National Collection of Type Cultures on 11 July 1990 as follows:

NCTC 12411: K12, TG1 E.coli containing plasmid pSVgptHuVHIconHMFG1-HuIgG1 (identified for the purposes of deposition simply as pSVgpt-HuVHHMFG1-HuIgG1)

NCTC 12412: K12, TG1 E.coli containing plasmid pSVneo-HuVkHMFG1-HuCk

7. Binding ability of the reshaped human antibodies

A useful way of demonstrating binding ability of the reshaped antibody is to show that it has a similar antibody dilution curve when binding to antigen adsorbed on a solid surface. Such curves were generated as follows, using the parent murine anti-HMFG antibody and a reshaped human antibody prepared by the foregoing procedure.

0.5ml of 10% w/v M280 tosyl activated magnetic beads (Dynal, Wirral, UK) were coupled to milk mucin (10^6 units as determined in an immunoassay for HMFG1 in which normal human serum registers 100-200 units per ml). Milk mucin

was prepared from human breast milk according to the method of Burchell et al (1987). The level of mucin was chosen to provide suitable activity for the assays in which the beads were used. The coupling was in 2.5ml of 0.5M borate buffer at pH 9.5 plus 2.5 ml of mucin in phosphate-buffered saline pH 7.2 (PBS) for 22hrs at 37°C with gentle rotation. Blocking of remaining active sites was accomplished by adding 1ml of 10% bovine serum albumen (BSA; Sigma) in PBSA (PBS + 0.02% sodium azide followed by a further 7 hr incubation at 37°C. The excess protein was washed away after using a samarium cobalt magnet to pellet the beads. Further washing was 3x in wash buffer (0.1M potassium phosphate pH 8.0, 0.1% Tween 20, 0.5% BSA) and 4x in rinse buffer (PBS + 0.1% BSA, 0.1% merthiolate). Beads were stored in rinse buffer at 10% w/v (estimated by dry weight analysis).

Antibody binding was measured from a series of doubling dilutions of antibody samples (prepared by weighing in critical cases). 50µl samples were incubated in replicate in microtitre wells with 50µl of 0.05% w/v suspension of beads in 1% BSA/PBSM (PBS + 0.01% merthiolate) at room temperature for 1 hr on a plate shaker. Small cobalt samarium magnets, embedded in a plastic base, were used to sediment the beads to the sides of the wells of the plate to allow liquid removal and washing once with 150µl PBSTM (PBSM + 0.15% Tween 20). This was followed by detection of bound antibody with 50µl of alkaline phosphatase coupled goat anti-human IgG (H+L) (Jackson) used at 1/1000 dilution in 1% BSA in PBSTM for 1 hr at room temperature. The beads were washed 3x in PBSTM. Colour development was with 200µl of nitro phenyl phosphate (Sigma alkaline phosphatase substrate tablets) in 1M diethanolamine buffer at pH 9.8. Optical densities were read in a Dynatech plate reader at 410nm after

transferring fixed volumes of supernatant (usually 150µl) to a flat bottom well microtitre plate. For examination of mouse antibodies the conjugate used was rabbit anti-mouse IgG (Sigma).

Antibody dilution curves for the murine and reshaped HMFG1 antibodies are shown in Figure 15. Maximum binding was determined with a large excess of antibody and negative controls had none. Antibody concentrations, in µg/ml, were determined by UV absorption measurements at 280nm. For both antibodies a dilution of 1 has been set equivalent to 1µg/ml. The two curves are similar, indicating a significant and useful level of binding effectiveness for the reshaped antibody of the invention.

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CLAIMS

1. A synthetic specific binding agent having specificity for human polymorphic epithelial mucin (PEM), conferred by the presence of one or more of the amino acid sequences:

i) Ala Tyr Trp Ile Glu

ii) Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr Asn Glu Lys Phe Lys Gly

iii) Ser Tyr Asp Phe Ala Trp Phe Ala Tyr

iv) Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

v) Trp Ala Ser Thr Arg Glu Ser

vi) Gln Gln Tyr Tyr Arg Tyr Pro Arg Thr

2 A reshaped human antibody, or a reshaped human antibody fragment, having specificity for human polymorphic epithelial mucin (PEM) conferred by the presence of one or more of the amino acid sequences:

i) Ala Tyr Trp Ile Glu

ii) Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr Asn Glu Lys Phe Lys Gly

iii) Ser Tyr Asp Phe Ala Trp Phe Ala Tyr

iv) Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

v) Trp Ala Ser Thr Arg Glu Ser

vi) Gln Gln Tyr Tyr Arg Tyr Pro Arg Thr

3. A reshaped human antibody or reshaped human antibody fragment according to claim 2, having at least one heavy-chain variable region incorporating the following CDRs:

CDR1: Ala Tyr Trp Ile Glu

CDR2: Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr Asn Glu Lys Phe Lys Gly

CDR3: Ser Tyr Asp Phe Ala Trp Phe Ala Tyr

4. A reshaped human antibody or reshaped human antibody fragment according to claim 2, having at least one light-chain variable region incorporating the following CDRs:

CDR1: Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

CDR2: Trp Ala Ser Thr Arg Glu Ser

CDR3: Gln Gln Tyr Tyr Arg Tyr Pro Arg Thr

5. A reshaped human antibody or reshaped human antibody fragment according to claim 2 and having at least one heavy-chain variable region according to claim 3 and at least one light-chain variable region according to claim 4.

6. A reshaped human antibody or reshaped human antibody fragment according to claim 2, incorporating at least one heavy-chain variable region comprising the entire amino acid sequence depicted in Figure 12 of the accompanying drawings.

7. A reshaped human antibody or reshaped human antibody fragment according to claim 2, incorporating at least one light-chain variable region comprising the entire amino acid sequence depicted in Figure 13 of the accompanying drawings.

8. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of the preceding claims, wherein the PEM is human milk fat globule (HMFG).

9. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, having specificity equivalent to that of the gamma-1, kappa anti-HMFG monoclonal antibody "HMFG1".

10. A stable host cell line producing a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment according to any one of claims 1 to 9, resulting from incorporation in the cell line of a foreign gene encoding the synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

11. A stable host cell line according to claim 10, wherein the foreign gene includes one or more of the nucleotide sequences:

i) GCC TAC TGG ATA GAG

ii) GAG ATT TTA CCT GGA AGT AAT AAT TCT AGA TAC AAT GAG AAG TTC AAG GGC

iii) TCC TAC GAC TTT GCC TGG TTT GCT TAC

iv) AAG TCC AGT CAG AGC CTT TTA TAT AGT AGC AAT CAA AAG ATC TAC TTG GCC

v) TGG GCA TCC ACT AGG GAA TCT

vi) CAG CAA TAT TAT AGA TAT CCT CGG ACG

12. A stable host cell line according to claim 10, wherein the foreign gene includes the entire nucleotide sequence depicted in Figure 12 of the accompanying drawings.

13. A stable host cell line according to claim 10, wherein the foreign gene includes the entire nucleotide sequence depicted in Figure 13 of the accompanying drawings.

14. A stable host cell line according to claim 10, wherein the foreign gene encodes:

a) at least one of the amino acid sequences:

i) Ala Tyr Trp Ile Glu

ii) Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr Asn Glu Lys Phe Lys Gly

iii) Ser Tyr Asp Phe Ala Trp Phe Ala Tyr

iv) Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys

Ile Tyr Leu Ala

v) Trp Ala Ser Thr Arg Glu Ser

vi) Gln Gln Tyr Tyr Arg Tyr Pro Arg Thr

and b) a protein framework that enables the encoded amino acid sequence when expressed to function as a CDR having specificity for PEM.

15. A stable host cell line according to claim 10, wherein the foreign gene encodes the entire amino acid sequence depicted in Figure 12 of the accompanying drawings.

16. A stable host cell line according to claim 10, wherein the foreign gene encodes the entire amino acid sequence depicted in Figure 13 of the accompanying drawings.

17. Plasmid pSVgpt-HuVHHMFG1-HuIgG1.

18. Plasmid pSVneo-HuVkHMFG1-HuCk.

19. Use of plasmid according to claim 17 or claim 18 in the production of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

20. E.coli NCTC 12411.

21. E.coli NCTC 12412.

22. A DNA sequence encoding a reshaped human antibody heavy-chain variable region having specificity for HMFG, as contained in E.coli NCTC 12411.

23. A DNA sequence encoding a reshaped human antibody light-chain variable region having specificity for HMFG, as contained in E.coli NCTC 12412.

24. A reshaped human antibody heavy-chain variable region having specificity for HMFG, producible by means of the expression vector contained in E.coli NCTC 12411.

25. A reshaped human antibody light-chain variable region having specificity for HMFG, producible by means of the expression vector contained in E.coli NCTC 12412.

26. A reshaped human antibody or reshaped human antibody fragment, comprising at least one variable region according to claim 24 or claim 25.

27. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of claims 1 to 9 or claim 26, linked to or incorporating an agent capable of retarding or terminating the growth of cancerous cells, or linked to an agent capable of being detected while inside the human body.

28. An injectable composition comprising a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to claim 27, in a pharmaceutically acceptable carrier.

29. Use of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of claims 1 to 9 or claim 26, for the

manufacture of a medicament for therapeutic application in the relief of cancer in humans, or for the manufacture of a diagnostic composition for in-vivo diagnostic application in humans.

30. Use of a synthetic binding agent, reshaped human antibody or reshaped human antibody fragment, according to claim 27, in a method of human cancer therapy or imaging.

Fig. 1.

MoVHHMFG1

```
                    5                  10                  15                  20
CAG GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG ATG AAG CCT GGG GCC TCA GTG AAG ATA     60
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Met Lys Pro Gly Ala Ser Val Lys Ile

                    25                  30       CDR1       35                  40
TCC TGC AAG GCT ACT GGC TAC ACA TTC AGT GCC TAC TGG ATA GAG TGG GTA AAG CAG AGG    120
Ser Cys Lys Ala Thr Gly Tyr Thr Phe Ser Ala Tyr Trp Ile Glu Trp Val Lys Gln Arg

                    45                  50      52  A           55       CDR2
CCT GGA CAT GGC CTT GAG TGG ATT GGA GAG ATT TTA CCT GGA AGT AAT AAT TCT AGA TAC    180
Pro Gly His Gly Leu Glu Trp Ile Gly Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr

60                      65                  70                  75
AAT GAG AAG TTC AAG GGC AAG GCC ACA TTC ACT GCT GAT ACA TCC TCC AAC ACA GCC TAC    240
Asn Glu Lys Phe Lys Gly Lys Ala Thr Phe Thr Ala Asp Thr Ser Ser Asn Thr Ala Tyr

80      82  A   B   C           85                  90                  95
ATG CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT GCC GTC TAT TAC TGT TCA AGG TCC TAC    300
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ser Arg Ser Tyr

 CDR3       100  A                  105                 110
GAC TTT GCC TGG TTT GCT TAC TGG GGC CAA GGG ACT CCG GTC ACT GTC TCT GCA           354
Asp Phe Ala Trp Phe Ala Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ala
```

Fig.2.

MoVkHMFG1

```
                    5                  10                  15                  20
GAC ATT GTG ATG TCA CAG TCT CCA TCC TCC CTA GCT GTG TCA GTT GGA GAG AAG GTT ACT   60
Asp Ile Val Met Ser Gln Ser Pro Ser Ser Leu Ala Val Ser Val Gly Glu Lys Val Thr

                25     27  A  B  C  D  E  F        30        CDR1
ATG AGC TGC AAG TCC AGT CAG AGC CTT TTA TAT AGT AGC AAT CAA AAG ATC TAC TTG GCC  120
Met Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

 35                 40                  45                  50        CDR2
TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC ACT AGG  180
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg

 55                 60                  65                  70
GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC GGT GGA TCT GGG ACA GAT TTC ACT CTC ACC  240
Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Gly Gly Ser Gly Thr Asp Phe Thr Leu Thr

 75                 80                  85                  90        CDR3
ATC AGC AGT GTG AAG GCT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGA TAT  300
Ile Ser Ser Val Lys Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Arg Tyr

 95                 100                 105
CCT CGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG                          342
Pro Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
```

Fig.3a

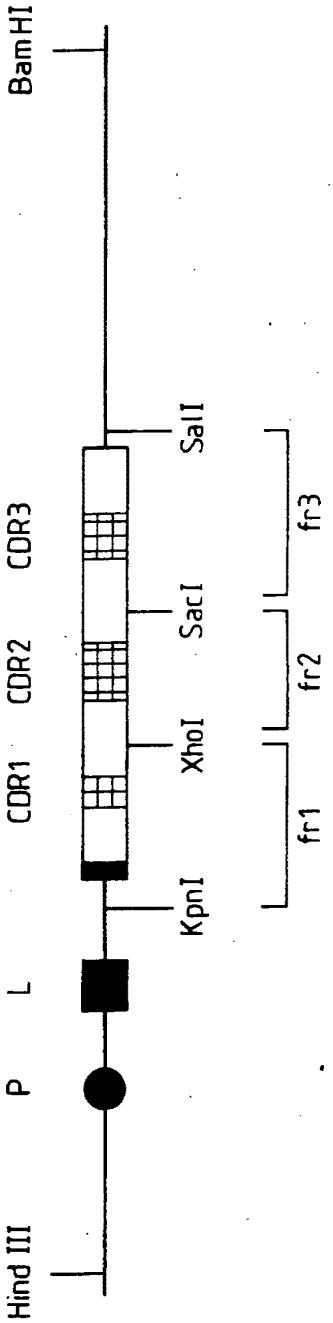


Fig. 3b

FRAGMENT 1

```
        10          20          30          40          50          60
acagtagcag gcttgaggaa agcttctata tatgggtacc aatgacatcc actttgcctt
tgtcatcgtc cgaactcctt tcgaagatat atacccatgg ttactgtagg tgaaacggaa

        70          80          90          100         110         120
tctctccaca gGTGTCCACT CCCAGGTGCA GCTGGTGCAG TCTGGGGCAG AGGTGAAAAA
agagaggtgt cCACAGGTGA GGGTCCACGT CGACCACGTC AGACCCCGTC TCCACTTTTT

       130         140         150         160         170         180
GCCTGGGGCC TCAGTGAAGG TGTCCTGCAA GGCTTCTGGC TACACCTTCA GTGCCTACTG
CGGACCCCGG AGTCACTTCC ACAGGACGTT CCGAAGACCG ATGTGGAAGT CACGGATGAC

       190         200         210         220         230         240
GATAGAGTGG GTGCGCCAGG CTCCAGGAAA GGGCCTCGAG TGGGTCGGAT CCAGGGAGAT
CTATCTCACC CACGCGGTCC GAGGTCCTTT CCCGGAGCTC ACCCAGCCTA GGTCCCTCTA
```

OLIGONUCLEOTIDES

CODE	LENGTH	5' ←--- SEQUENCE ---→ 3'
VHHM1A	(32)	agc ttc tat ata tgg gta cca atg aca tcc ac
VHHM1B	(33)	ttt gcc ttt ctc tcc aca gGT GTC CAC TCC CAG
VHHM1C	(36)	GTG CAG CTG GTG CAG TCT GGG GCA GAG GTG AAA AAG
VHHM1D	(33)	CCT GGG GCC TCA GTG AAG GTG TCC TGC AAG GCT
VHHM1E	(36)	TCT GGC TAC ACC TTC AGT GCC TAC TGG ATA GAG TGG
VHHM1F	(37)	GTG CGC CAG GCT CCA GGA AAG GGC CTC GAG TGG GTC G
VHHM1G	(40)	gag aaa ggc aaa gtg gat gtc att ggt acc cat ata tag a
VHHM1H	(36)	CTG CAC CAG CTG CAC CTG GGA GTG GAC ACc tgt gga
VHHM1I	(33)	TGA GGC CCC AGG CTT TTT CAC CTC TGC CCC AGA
VHHM1J	(33)	GGT GTA GCC AGA AGC CTT GCA GGA CAC CTT CAC
VHHM1K	(36)	AGC CTG GCG CAC CCA CTC TAT CCA GTA GGC ACT GAA
VHHM1L	(29)	GAT CCG ACC CAC TCG AGG CCC TTT CCT GG

POSITIVE STRING:

```
VHHM1A  : (21-52)
VHHM1B  : (53-85)
VHHM1C  : (86-121)
VHHM1D  : (122-154)
VHHM1E  : (155-190)
VHHM1F  : (191-227)
```

NEGATIVE STRING:

```
VHHM1G  : (25-64)
VHHM1H  : (65-100)
VHHM1I  : (101-133)
VHHM1J  : (134-166)
VHHM1K  : (167-202)
VHHM1L  : (203-231)
```

Fig. 3c.

FRAGMENT 2

```
        10          20          30          40          50          60
GACAGCCGTA GAGTGGGTGC AAGCTTCTCC AGGACTCGAG TGGGTCGGAG AGATTTTACC
CTGTCGGCAT CTCACCCACG TTCGAAGAGG TCCTGAGCTC ACCCAGCCTC TCTAAAATGG

        70          80          90          100         110         120
TGGAAGTAAT AATTCTAGAT ACAATGAGAA GTTCAAGGGC CGAGTGACAG TCACTAGAGA
ACCTTCATTA TTAAGATCTA TGTTACTCTT CAAGTTCCCG GCTCACTGTC AGTGATCTCT

       130         140         150         160         170         180
CACATCCACA AACACAGCCT ACATGGAGCT CAGCAGCCTG AGGATCCAGC AGCCTGAGGT
GTGTAGGTGT TTGTGTCGGA TGTACCTCGA GTCGTCGGAC TCCTAGGTCG TCGGACTCCA
```

OLIGONUCLEOTIDES

CODE	LENGTH	5' ←--- SEQUENCE ---→ 3'
VHHM2A	(25)	AGC TTC TCC AGG ACT CGA GTG GGT C
VHHM2B	(27)	GGA GAG ATT TTA CCT GGA AGT AAT AAT
VHHM2C	(39)	TCT AGA TAC AAT GAG AAG TTC AAG GGC CGA GTG ACA GTC
VHHM2D	(30)	ACT AGA GAC ACA TCC ACA AAC ACA GCC TAC
VHHM2E	(20)	ATG GAG CTC AGC AGC CTG AG
VHHM2F	(36)	AGG TAA AAT CTC TCC GAC CCA CTC GAG TCC TGG AGA
VHHM2G	(39)	GCC CTT GAA CTT CTC ATT GTA TCT AGA ATT ATT ACT TCC
VHHM2H	(24)	TGT GTC TCT AGT GAC TGT CAC TCG
VHHM2I	(42)	GAT CCT CAG GCT GCT GAG CTC CAT GTA GGC TGT GTT TGT GGA

POSITIVE STRING:

```
VHHM2A   : (22-46)
VHHM2B   : (47-73)
VHHM2C   : (74-112)
VHHM2D   : (113-142)
VHHM2E   : (143-162)
```

NEGATIVE STRING:

```
VHHM2F   : (26-61)
VHHM2G   : (62-100)
VHHM2H   : (101-124)
VHHM2I   : (125-166)
```

Fig. 3d

FRAGMENT 3

```
        10          20          30          40          50          60
CACATCCACA AGCTTAAACA CAGCCGAGCT CAGCAGCCTG AGGTCTGAGG ACACAGCCGT
GTGTAGGTGT TCGAATTTGT GTCGGCTCGA GTCGTCGGAC TCCAGACTCC TGTGTCGGCA

        70          80          90         100         110         120
CTATTACTGT GCAAGATCCT ACGACTTTGC CTGGTTTGCT TACTGGGGCC AAGGGACTCT
GATAATGACA CGTTCTAGGA TGCTGAAACG GACCAAACGA ATGACCCCGG TTCCCTGAGA

       130         140         150         160         170         180
GGTCACAGTC TCCTCAggtg agtccttaca acctctctct tctattcagt cgacatagat
CCAGTGTCAG AGGAGTccac tcaggaatgt tggagagaga agataagtca gctgtatcta

       190
acgtggatcc
tgcacctagg
```

OLIGONUCLEOTIDES

CODE	LENGTH	5' ←--- SEQUENCE ---→ 3'
VHHM3A	(39)	AGC TTA AAC ACA GCC GAG CTC AGC AGC CTG AGG TCT GAG
VHHM3B	(27)	GAC ACA GCC GTC TAT TAC TGT GCA AGA
VHHM3C	(39)	TCC TAC GAC TTT GCC TGG TTT GCT TAC TGG GGC CAA GGG
VHHM3D	(39)	ACT CTG GTC ACA GTC TCC TCA ggt gag tcc tta caa cct
VHHM3E	(31)	ctc tct tct att cag tcg aca tag ata cgt g
VHHM3F	(17)	GAG CTC GGC TGT GTT TA
VHHM3G	(33)	ATA GAC GGC TGT GTC CTC AGA CCT CAG GCT GCT
VHHM3H	(39)	GTA AGC AAA CCA GGC AAA GTC GTA GGA TCT TGC ACA GTA
VHHM3I	(36)	acc TGA GGA GAC TGT GAC CAG AGT CCC TTG GCC CCA
VHHM3J	(29)	tga ata gaa gag aga ggt tgt aag gac tc
VHHM3K	(21)	gat cca cgt atc tat gtc gac

POSITIVE STRING:

VHHM3A : (11-49)
VHHM3B : (50-76)
VHHM3C : (77-115)
VHHM3D : (116-154)
VHHM3E : (155-185)

NEGATIVE STRING:

VHHM3F : (15-31)
VHHM3G : (32-64)
VHHM3H : (65-103)
VHHM3I : (104-139)
VHHM3J : (140-168)
VHHM3K : (169-189)

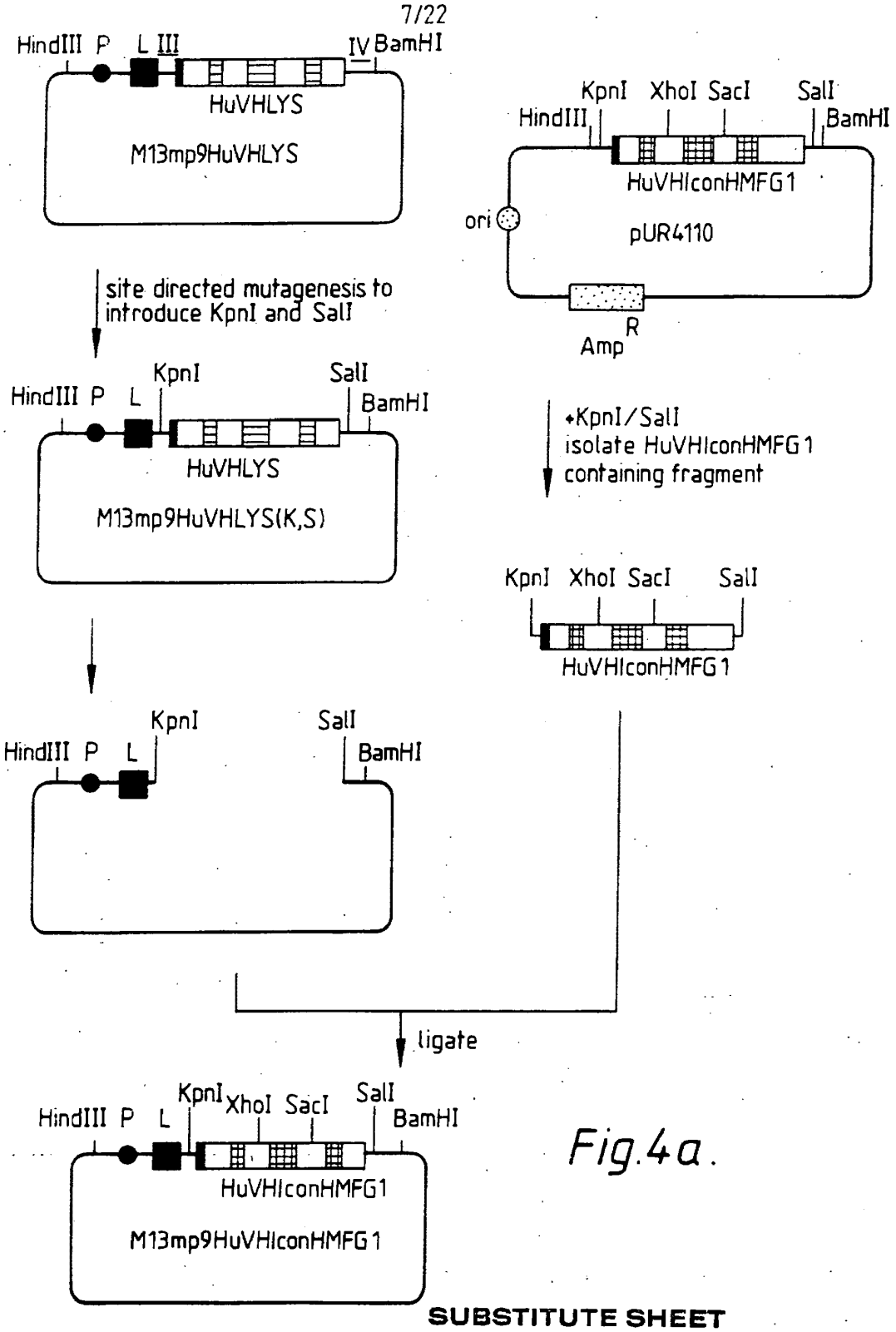


Fig.4a.

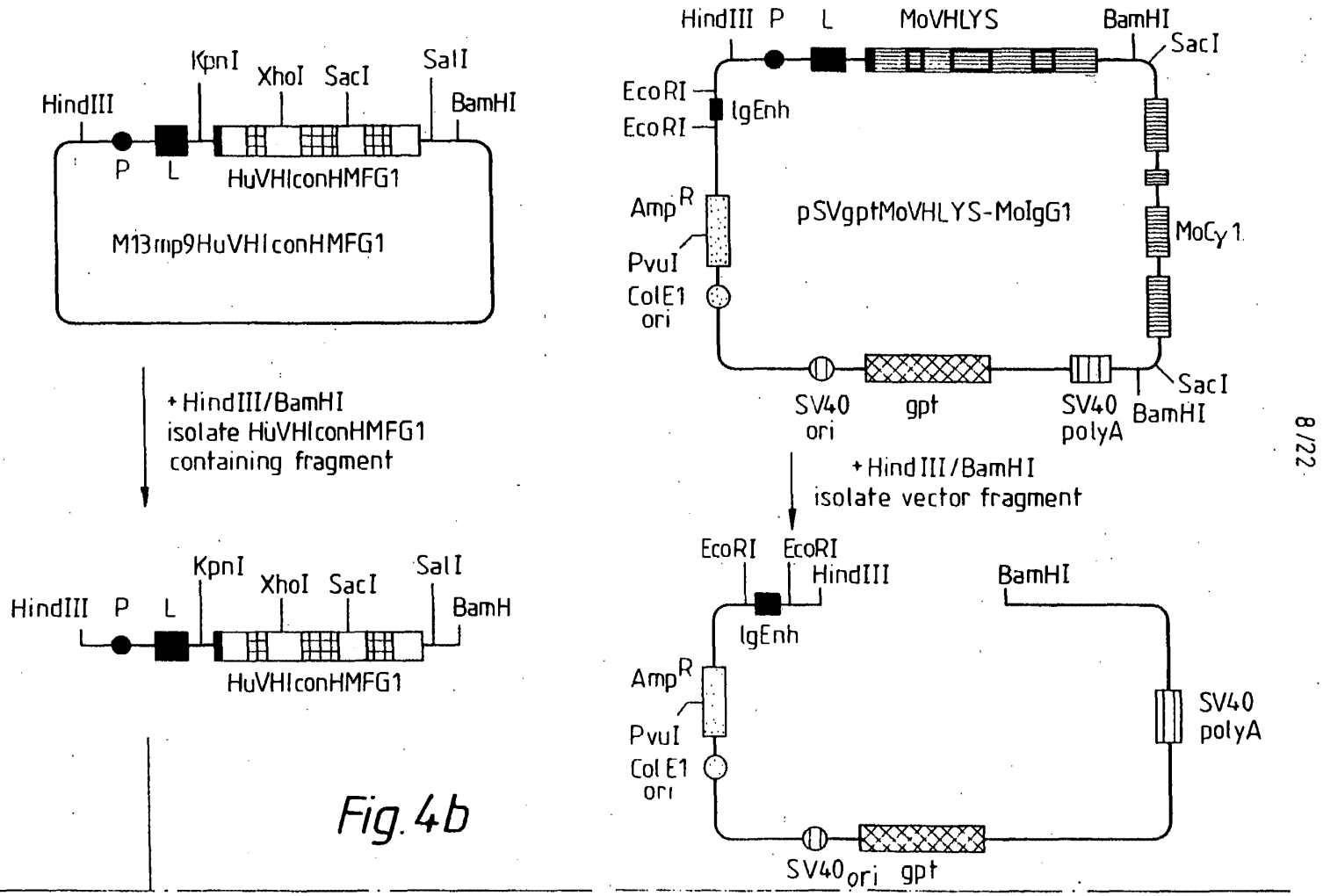
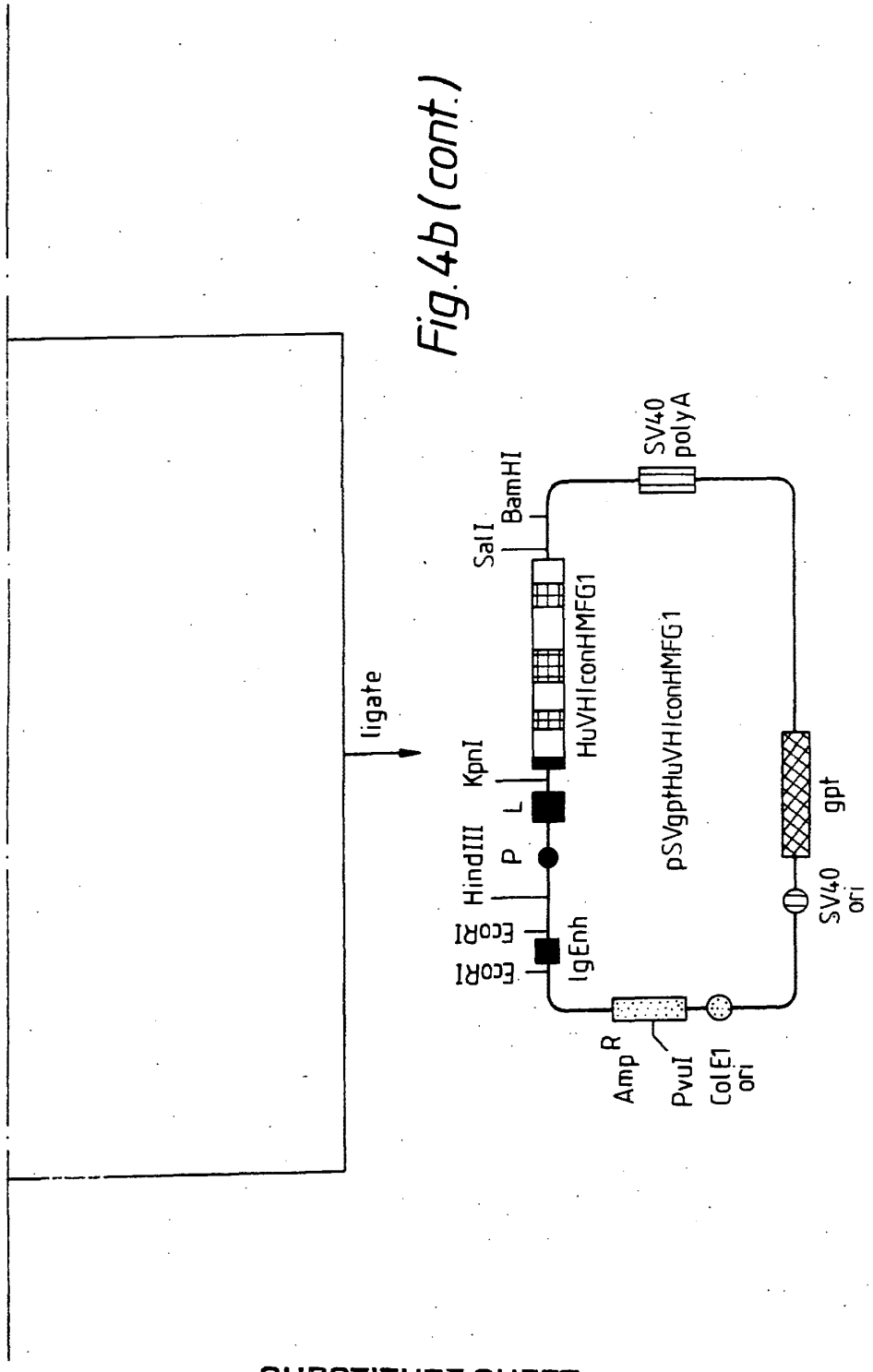


Fig. 4b

Fig.4b (cont.)



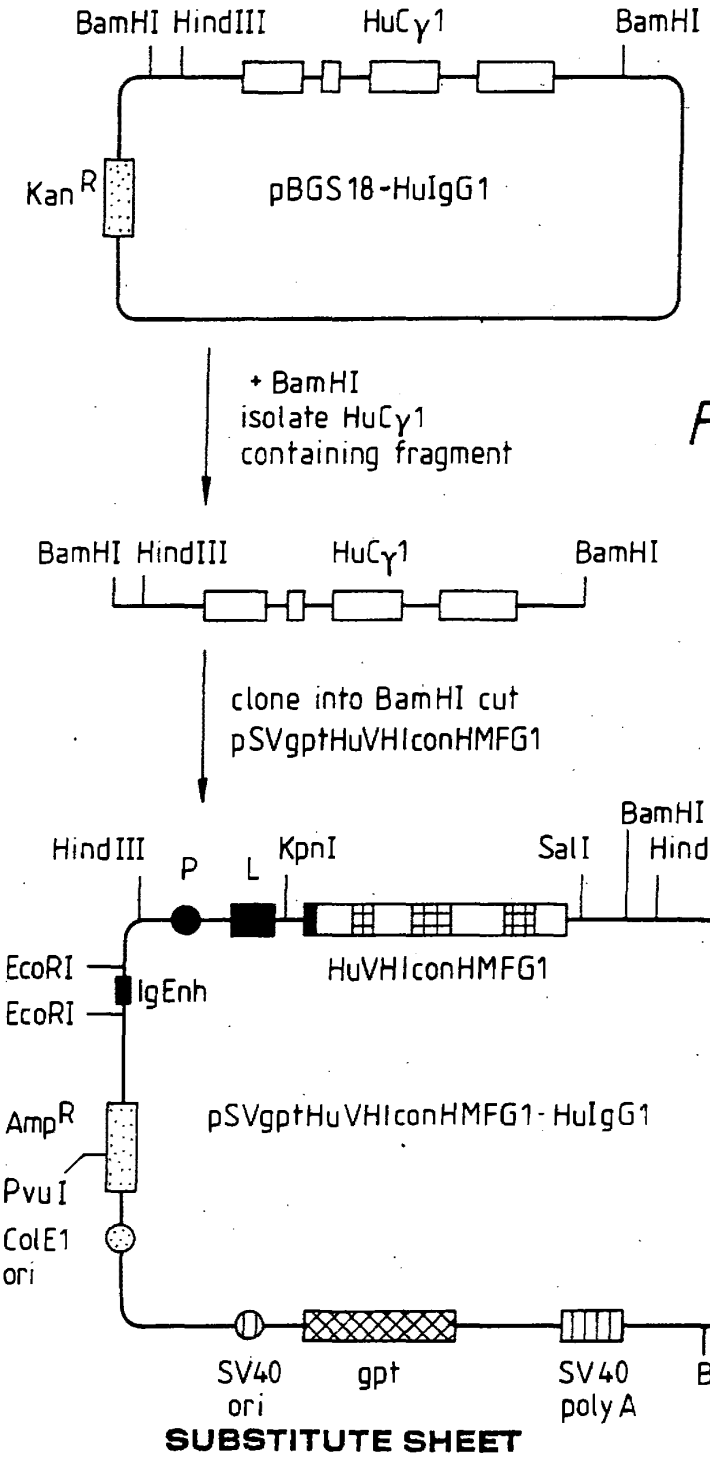


Fig. 4c

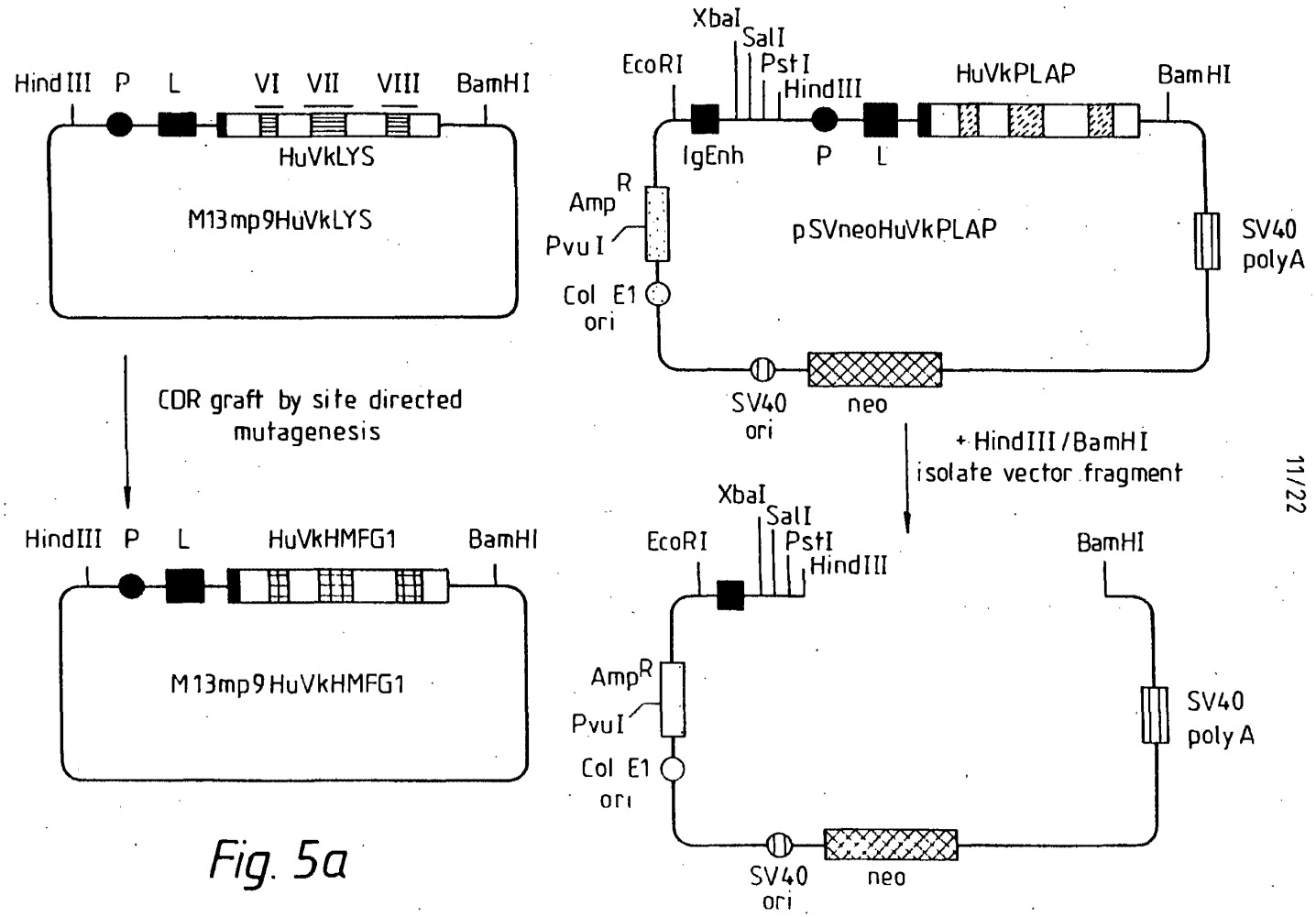
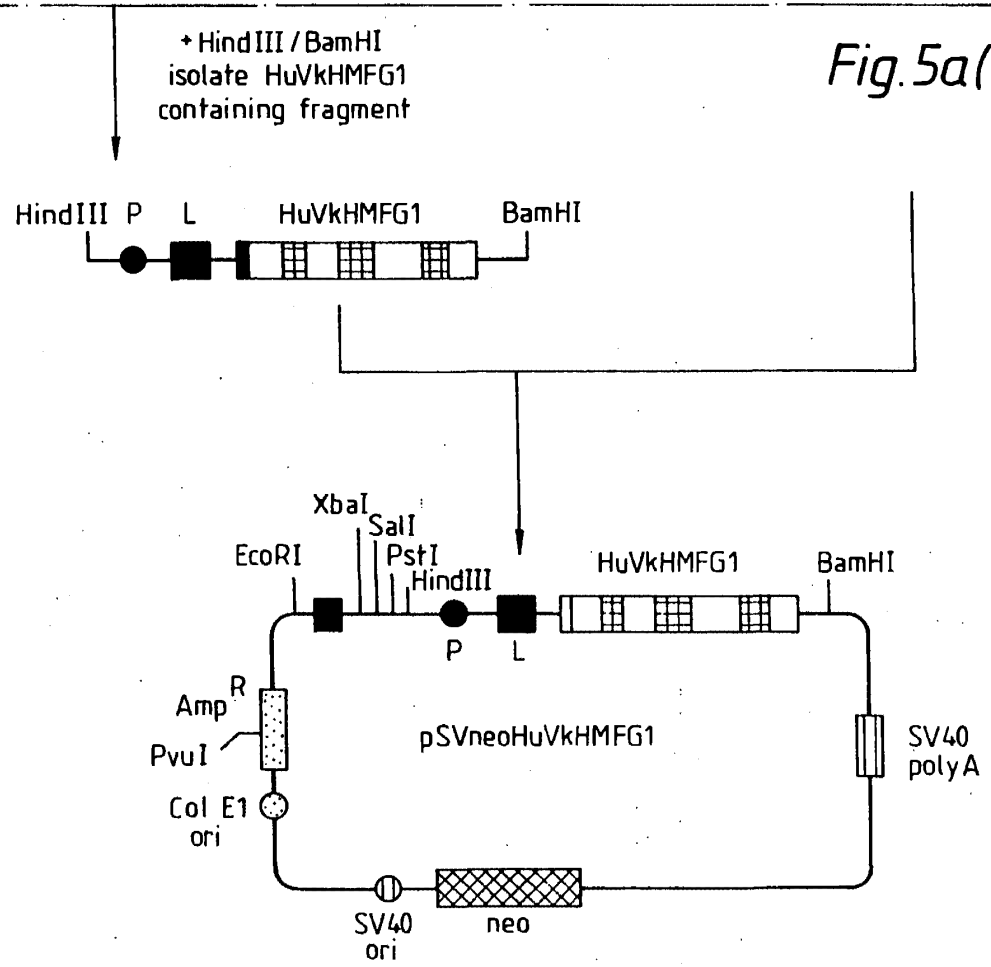

HindIII P L VI VII VIII BamHI
HuVkLYS
M13mp9HuVkLYS
CDR graft by site directed mutagenesis
HindIII P L HuVkHMFG1 BamHI
M13mp9HuVkHMFG1
XbaI SalI
EcoRI PstI
HindIII
HuVkPLAP
BamHI
IgEnh P L
Amp R
Pvu I
pSVneoHuVkPLAP
SV40 polyA
Col E1 ori
SV40 ori
neo
+ HindIII/BamHI isolate vector fragment
XbaI SalI
EcoRI PstI
HindIII
BamHI
Amp R
Pvu I
Col E1 ori
SV40 poly A
SV40 ori
neo

Fig. 5a

Fig. 5a (cont.)



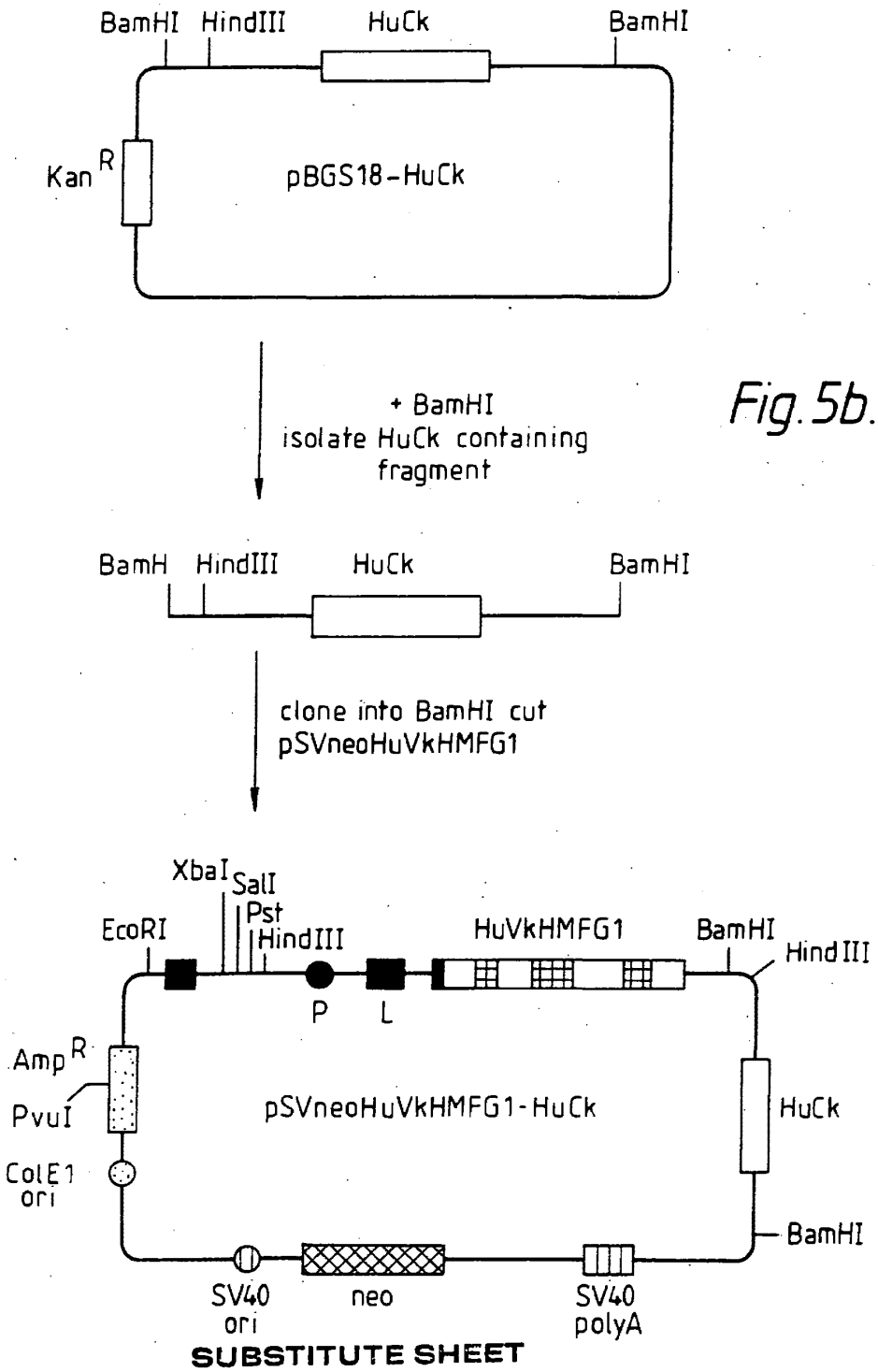


Fig. 5b.

Fig.6.

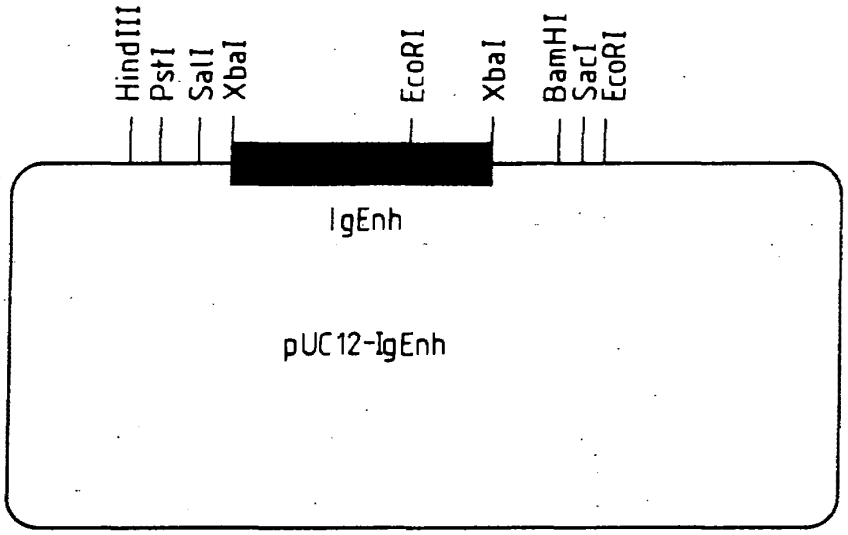


Fig. 7.

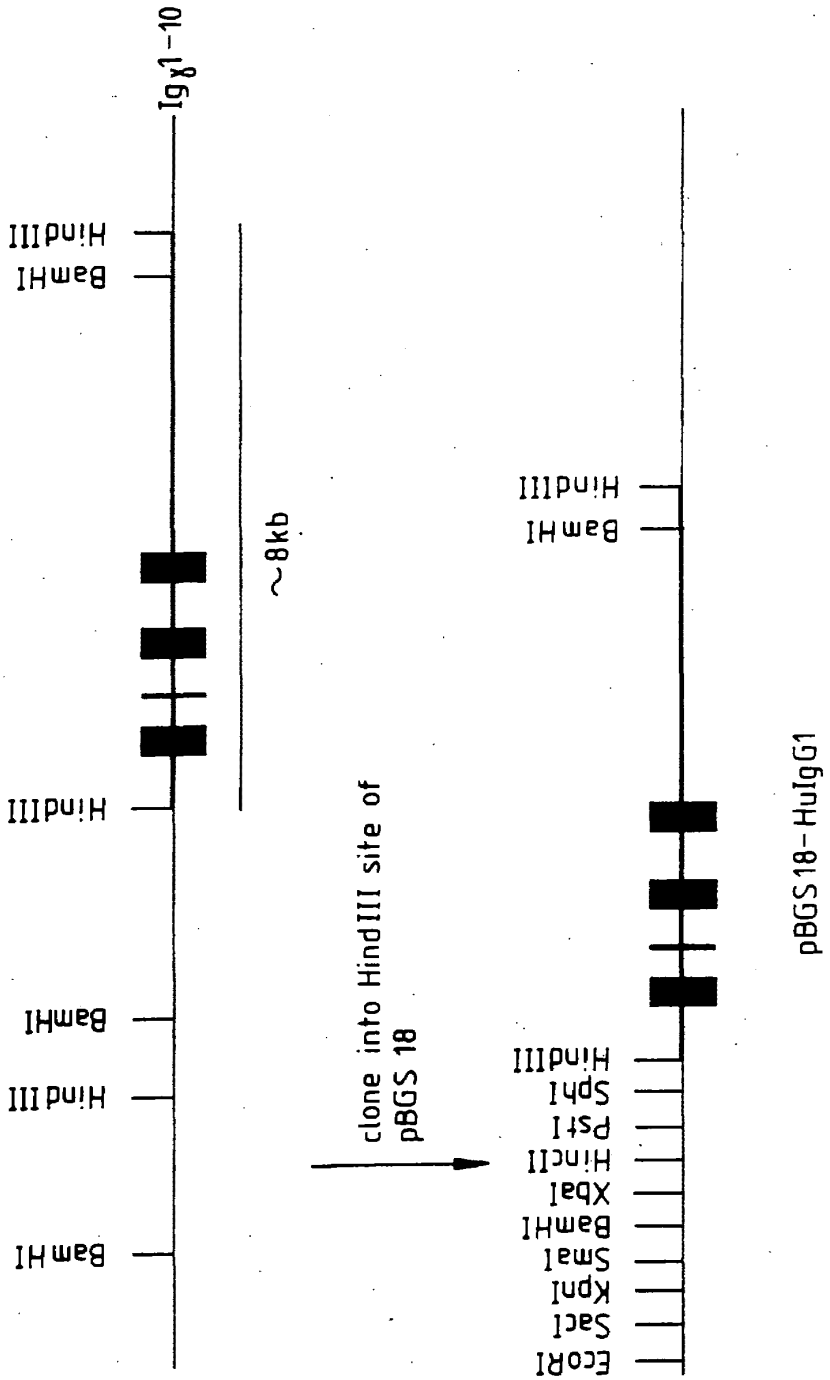
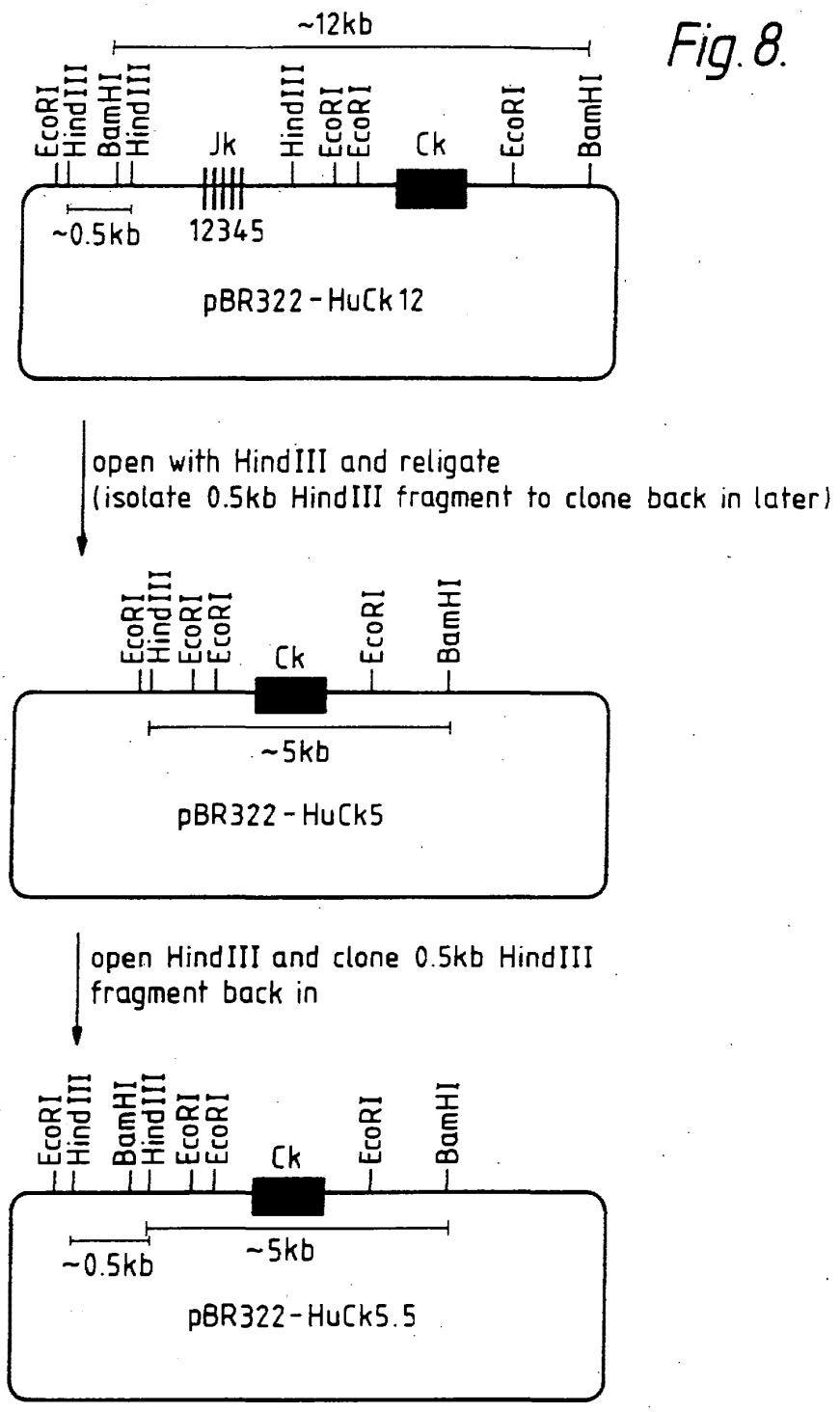


Fig. 8.



Subclone HuCk containing BamHI fragment in pBGS18-BamHI gives: pBGS18-HuCk

SUBSTITUTE SHEET

Fig. 9.

Oligonucleotides used for cloning variable region genes.

I : mouse constant gamma1 primer

5' GAT AGA CAG ATG GGG GTG TCG TTT 3'

II : mouse constant kappa primer

5' AGA TGG ATA CAG TTG GTG CAG CAT 3'

Fig.10.

Oligonucleotides used to introduce KpnI and SalI in M13mp9HuVHLYS.

III : to introduce a KpnI in the HuVH leader intron

5' TGT CAT TGG TAC CCA TAT 3'

IV : to introduce a SalI 5' of the HuVHLYS gene

5' AAA TCT ATG TCG ACT GAA TAG 3'

Fig.11.

Oligonucleotides used for grafting of VkHMFG1 CDRs onto human kappa chain framework regions.

VI : VkHMFG1-CDR1

5' CTG CTG GTA CCA GGC CAA GTA GAT CTT TTG ATT GCT ACT ATA

TAA AAG GCT CTG ACT GGA CTT ACA GGT GAT GGT 3'

VII : VkHMFG1-CDR2

5' GCT TGG CAC ACC AGA TTC CCT AGT GGA TGC CCA GTA GAT

CAG CAG 3'

VIII : VkHMFG1-CDR3

5' CCC TTG GCC GAA CGT CCG AGG ATA TCT ATA ATA TTG CTG

GCA GTA GTA GGT 3'

Fig. 12.

HuVHIconHMFG1

```
                    5                  10                  15                  20
CAG GTG CAG CTG GTG CAG TCT GGG GCA GAG GTG AAA AAG CCT GGG GCC TCA GTG AAG GTG     60
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val

                   25                  30        CDR1      35                  40
TCC TGC AAG GCT TCT GGC TAC ACC TTC AGT GCC TAC TGG ATA GAG TGG GTG CGC CAG GCT    120
Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Ala Tyr Trp Ile Glu Trp Val Arg Gln Ala

                   45                  50      52  A           55        CDR2
CCA GGA AAG GGC CTC GAG TGG GTC GGA GAG ATT TTA CCT GGA AGT AAT AAT TCT AGA TAC    180
Pro Gly Lys Gly Leu Glu Trp Val Gly Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr

 60                  65                  70                  75
AAT GAG AAG TTC AAG GGC CGA GTG ACA GTC ACT AGA GAC ACA TCC ACA AAC ACA GCC TAC    240
Asn Glu Lys Phe Lys Gly Arg Val Thr Val Thr Arg Asp Thr Ser Thr Asn Thr Ala Tyr

 80      82  A   B   C           85                  90                  95
ATG GAG CTC AGC AGC CTG AGG TCT GAG GAC ACA GCC GTC TAT TAC TGT GCA AGA TCC TAC    300
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Ser Tyr

 CDR3        100  A                 105                 110
GAC TTT GCC TGG TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACA GTC TCC TCA            354
Asp Phe Ala Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
```

SUBSTITUTE SHEET

Fig. 13.

HuVkHMFG1

```
                    5                   10                  15                  20
GAC ATC CAG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT GAC AGA GTG ACC      60
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr

                   25      27  A   B   C   D   E   F       30        CDR1
ATC ACC TGT AAG TCC AGT CAG AGC CTT TTA TAT AGT AGC AAT CAA AAG ATC TAC TTG GCC     120
Ile Thr Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

35                  40                  45                  50        CDR2
TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC TGG GCA TCC ACT AGG     180
Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg

55                  60                  65                  70
GAA TCT GGT GTG CCA AGC AGA TTC AGC GGT AGC GGT AGC GGT ACC GAC TTC ACC TTC ACC     240
Glu Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr

75                  80                  85                  90        CDR3
ATC AGC AGC CTC CAG CCA GAG GAC ATC GCC ACC TAC TAC TGC CAG CAA TAT TAT AGA TAT     300
Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Arg Tyr

95                  100                 105
CCT CGG ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGT                           342
Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
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Fig. 14.

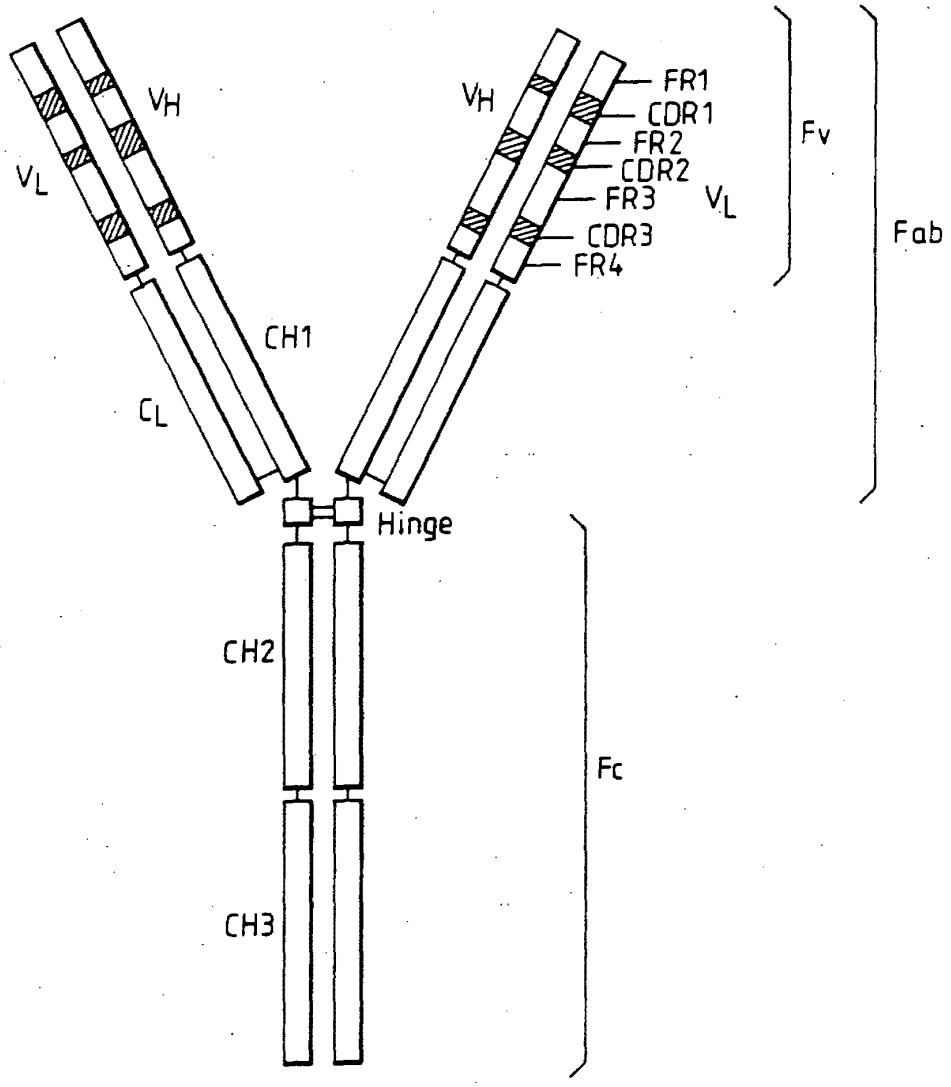
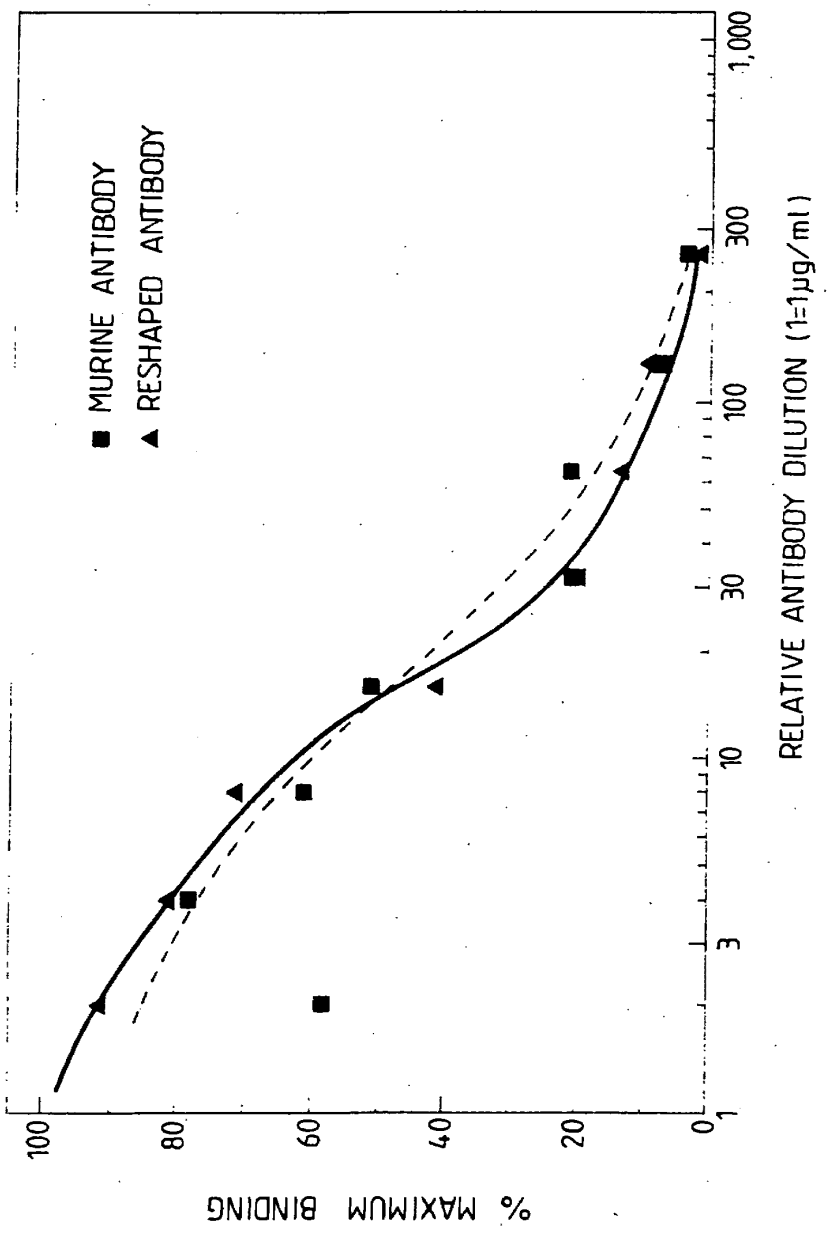


Fig. 15.



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01511

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 K 15/28 C 12 P 21/08 C 12 N 1/21
C 12 N 15/13 A 61 K 39/395

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 P A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	NATURE, vol. 332, 24th March 1988, (London, GB), L. RIECHMANN et al.: "Reshaping human antibodies for therapy", pages 323-327, see page 325, right-hand column, line 5 - page 326, left-hand column, line 40 (cited in the application) ---	1-30
Y	EP,A,0369816 (THE UNIVERSITY OF MELBOURNE) 23 May 1990, see the whole document (cited in the application) ---	1-30
Y	WO,A,8907268 (JOHN MUIR CANCER & AGING INSTITUTE) 10 August 1989, see the whole document ---	1-30
Y	WO,A,9005142 (IMPERIAL CANCER RESEARCH TECHNOLOGY LTD) 17 May 1990, see claims 14-23 --- -/-	1-30

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02-12-1991	21. 01. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	miss T. MØRTENSEN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8809344 (CREATIVE BIOMOLECULES, INC.) 1 December 1988, see the claims ---	1-30
P,Y	WO,A,9107500 (UNILEVER PLC) 30 May 1991, see the whole document ---	1-30
P,Y	WO,A,9012319 (JOHN MUIR CANCER & AGING INSTITUTE) 18 October 1990, see the claims -----	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 30 is directed to a method of treatment of, and the diagnostic method practised on, the human body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9101511 SA 51125

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/12/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0369816	23-05-90	CA-A- 2003211	17-05-90
WO-A- 8907268	10-08-89	AU-A- 3049189	25-08-89
		EP-A- 0401247	12-12-90
		JP-T- 3503120	18-07-91
WO-A- 9005142	17-05-90	EP-A- 0442926	28-08-91
WO-A- 8809344	01-12-88	AU-B- 612370	11-07-91
		AU-A- 1804988	21-12-88
		EP-A- 0318554	07-06-89
		JP-T- 2500329	08-02-90
WO-A- 9107500	30-05-91	AU-A- 6736990	13-06-91
		EP-A- 0429242	29-05-91
WO-A- 9012319	18-10-90	None	

#36 (Supp) 10/7/97 OFFICIAL PLEASE ENTER P.N. 10-7-97

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1816 Examiner: P. Nolan CERTIFICATE OF HAND DELIVERY I hereby certify that this correspondence is being delivered to Receptionist, Group 1800 of the United States Patent and Trademark Office, Washington, D.C. 20231 on October 7, 1997 R. H. Mitchell Printed Name R. H. Mitchell

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants submit herewith patents, publications or other information (attached hereto and listed on the attached Form PTO-1449) of which they are aware, which they believe may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 CFR §1.56.

This Information Disclosure Statement:

(a) [ ] accompanies the new patent application submitted herewith. 37 CFR §1.97(a).

(b) [ ] is filed within three months after the filing date of the application or within three months after the date of entry of the national stage of a PCT application as set forth in 37 CFR§1.491.

(c) [ ] as far as is known to the undersigned, is filed before the mailing date of a first Office action on the merits.

(d) [X] is filed after the first Office Action and more than three months after the application's filing date or PCT national stage date of entry filing but, as far as is known to the undersigned, prior to the mailing date of either a final rejection or a notice of allowance, whichever occurs first, and is accompanied by either the fee ($230) set forth in 37 CFR §1.17(p) **or** a certification as specified in 37 CFR §1.97(e), as checked below. Should any fee be due, the U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $220.00 to cover the cost of this Information Disclosure Statement. Any deficiency or overpayment should be charged or credited to this deposit account. **A duplicate of this sheet is enclosed.**

(e) [ ] is filed after the mailing date of either a final rejection or a notice of allowance, whichever occurred first, and is accompanied by the fee ($130) set forth in 37 CFR §1.17(i)(1) **and** a certification as specified in 37 CFR §1.97(e), as checked below. **This document is to be considered as a petition requesting consideration of the information disclosure statement.** The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $130.00 to cover the cost of this Information Disclosure Statement. Any deficiency or overpayment should be charged or credited to this deposit account. **A duplicate of this sheet is enclosed.**

[If either of boxes (d) or (e) is checked above, the following "certification" under 37 CFR §1.97(e) may need to be completed.] The undersigned certifies that:

[ ] Each item of information contained in the information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement.

[ ] No item of information contained in this information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application or, to the knowledge of the undersigned after making reasonable inquiry, was known to any individual designated in 37 CFR §1.56(c) more than three months prior to the filing of this information disclosure statement.

A list of the patent(s) or publication(s) is set forth on the attached Form PTO-1449 (Modified).

A copy of the items on PTO-1449 is supplied herewith:

[x] each [ ] none [ ] only those listed below:

Those patent(s) or publication(s) which are marked with an asterisk (*) in the attached PTO-1449 form are not supplied because they were previously cited by or submitted to the Office in a prior application Serial No. ___, filed _____and relied upon in this application for an earlier filing date under 35 USC §120.

A concise explanation of relevance of the items listed on PTO-1449 is:

[x] not given

[ ] given for each listed item

[ ] given for only non-English language listed item(s) [Required]

[ ] in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references.

The Examiner is reminded that a "concise explanation of the relevance" of the submitted prior art "may be nothing more than identification of the particular figure or paragraph of the patent or publication which has some relation to the claimed invention," MPEP §609.

While the information and references disclosed in this Information Disclosure Statement may be "material" pursuant to 37 CFR §1.56, it is not intended to constitute an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

In accordance with 37 CFR §1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR §1.56(a) exists. It is submitted that the Information Disclosure Statement is in compliance with 37 CFR §1.98 and MPEP §609 and the Examiner is respectfully requested to consider the listed references.

Respectfully submitted,

GENENTECH, INC.

Date: October 6, 1997

By: _____

Wendy M. Lee
Reg. No. 40,378

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

Duplicate of DS received at 04/17/95

Sheet 1 of 4

FORM PTO-1449 — U.S. Dept. of Commerce, Patent and Trademark Office

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter and Presta	
Filing Date: 17 Nov 1993	Group: ~~1806~~ 1816

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
TBS PN	1	4,816,567	~~28.03.89~~ 3-28-89	Cabilly et al.			

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Examiner Initials		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
TBS PN	2	0 239 400	~~9-30-87~~ ~~30.09.87~~	EPO				
	3	0 620 276	~~10-19-94~~ ~~19.10.94~~	EPO				
	4	WO 89/01783	~~3-9-89~~ ~~09.03.89~~	PCT				
	5	WO 89/06692	~~7-27-89~~ ~~27.07.89~~	PCT				
	6	WO 90/07861	~~7-26-90~~ ~~26.07.90~~	PCT				
	7	WO 91/09967	~~7-11-91~~ ~~11.07.91~~	PCT				
	8	WO 92/22653	~~12-23-92~~ ~~23.12.92~~	PCT				
PN	9	WO 93/02191	~~2-4-93~~ ~~04.02.93~~	PCT				

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Patricia A. Duffy	12/16/96

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

M. T. DAVIS 12/05-101

USCOMM-DC 80-398.

FORM PTO-1449 — U.S. Dept. of Commerce, Patent and Trademark Office

LIST OF DISCLOSURES CITED BY APPLICANT
(Use several sheets if necessary)

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter and Presta	
Filing Date: 17 Nov 1993	Group: 1806

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Examiner: Adams — Date Considered: 10/25/95

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M.T. DAVIS 12/05/01

USCOMM-DC 80-398.

FORM PTO-1449	U.S. Dept. of Commerce Patent and Trademark Office	Atty Docket No. P0709P1	Serial No. 08/146,206
LIST OF DISCLOSURES CITED BY APPLICANT (Use several sheets if necessary)		Applicant Carter and Presta MAILED	
		Filing Date 17 Nov 1993	Group ~~1806~~ 1816 DEC 20 1995

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Examiner	Date Considered
Patrick J. Nolan	12/16/96

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FORM PTO-1449

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Patent and Trademark Office

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter and Presta	
Filing Date: 17 Nov 1993	Group: 1806

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Examiner: Adams

Date Considered: 10/25/95

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M. T. DAVIS 12/05/01

USCOMM-DC 80-398.

Patent Docket P0709P1 #32

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1816 Examiner: P. Nolan **CERTIFICATE OF HAND DELIVERY** I hereby certify that this correspondence is being delivered to Receptionist, Group 1800 of the United States Patent and Trademark Office, Washington, D.C. 20231 on October 7, 1997 R. H. Mitchell Printed Name: R. H. Mitchell

AMENDMENT TRANSMITTAL

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Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated as shown below.

	Claims Remaining After Amendment		Highest No. Previously Paid For	Present Extra	Rate	Additional Fees
Total	35	-	31	4	x 88 =	$88.00
Independent	8	-	10	0	x 80 =	$0.00
__ First Presentation of Multiple Dependent Claims					+ 260 =	
					Total Fee Calculation	$88.00

_____
X
_____

No additional fee is required.
The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $88.00. **A duplicate copy of this transmittal is enclosed.**
Petition for Extension of Time is enclosed.

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR 1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. **A duplicate copy of this sheet is enclosed.**

Respectfully submitted,
GENENTECH, INC.

Date: October 6, 1997

By: _____
Wendy M. Lee
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#32/8
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10/7/97

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: 17 November 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1816 Examiner: P. Nolan CERTIFICATE OF HAND DELIVERY I hereby certify that this correspondence is being delivered to Receptionist, Group 1800 of the United States Patent and Trademark Office, Washington, D.C. 20231 on October ___, 1997 Printed Name:

SUPPLEMENTAL AMENDMENT UNDER 37 C.F.R. §1.111

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Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants respectfully request reconsideration of the above-identified application in view of the following amendments and remarks.

IN THE SPECIFICATION:

On page 8, lines 25-27 and page 15, lines 23-24, please replace the sequence in its entirety with the following sequence --

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENGSDTYYADS
VKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCARDRGGAVSYFDVWGQGTLVTVSS--

On page 9, line 30, please replace "huκI" with --huIII--.

IN THE CLAIMS:

10/10/1997 PSTAMBAC 00000021 DA8:070630 08146206
01 FC:103 08.00 CH

10. (Three times amended) A humanized antibody variable domain having a non-human Complementarity Determining Region (CDR) incorporated into a human antibody variable domain, wherein an amino acid residue has been substituted for the human amino acid residue at a site selected from the group consisting of:

4L, [36L], 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, [70L,] 73L, 85L, [87L,] 98L, 2H,

4H, [24H,] 36H, [37H,] 39H, 43H, 45H, [49H, 68H,] 69H, 70H, [73H,] 74H, 75H, 76H, 78H and 92H.

Please add the following claims:

--39. A humanized heavy chain variable domain comprising FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, wherein FR1-4 comprise the four framework regions of a consensus human variable domain of a human heavy chain immunoglobulin subgroup and CDR1-3 comprise the three complementarity determining regions (CDRs) of a nonhuman import antibody, and further wherein consensus human framework region (FR) residues have been replaced by nonhuman import residues where the FR residue (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) comprises a glycosylation site which affects the antigen binding or affinity of the antibody; or (d) participates in the V_L - V_H interface.

40. The humanized heavy chain variable domain of claim 39 wherein the human heavy chain immunoglobulin subgroup is V_H subgroup III.

41. The humanized heavy chain variable domain of claim 40 wherein:
FR1 of the consensus human variable domain comprises the amino acid sequence:
EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO:27);
FR2 of the consensus human variable domain comprises the amino acid sequence:
WVRQAPGKGLEWVA (SEQ ID NO:28);
FR3 of the consensus human variable domain comprises the amino acid sequence:
RFTISRDDSKNTLYLQMNSLRAEDTAVYYCAR (SEQ ID NO:29); and
FR4 of the consensus human variable domain comprises the amino acid sequence:
WGQGTLVTVSS (SEQ ID NO:30).

42. The humanized antibody of claim 22 which lacks immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient.--

REMARKS

A. Amendments

The undersigned confirms having met with Examiners Nolan and Eisenschenk in the interview 7/23/97 and takes this opportunity to thank the Examiners for the courtesies extended in the interview. Claims 39-41 have been added herein which use language as proposed by Examiner Nolan in the interview. Independent claim 39 is similar to a combination of presently pending claims 22 and 23. Basis for the language "FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, wherein FR1-4 comprise the four framework regions of a consensus human variable domain of a human heavy chain immunoglobulin subgroup and CDR1-3 comprise the three complementarity determining regions (CDRs) of a nonhuman import antibody" in claim 39 is found on page 1, lines 28-30 and page 25, lines 28-29, for example. Claim 40 finds specification basis on at least page 15, line 18. Claim 41 finds specification support in Figure 1B with respect to the framework regions of the HUV_HIII consensus sequence therein. Claim 42 has also been added and finds specification basis on at least page 60, lines 25-32 and page 70, lines 6-8. With respect to the amendments to the specification, the sequence on pages 8 and 15 has been corrected (see Section B of this amendment) and the typographical error with respect to the Fig. 5 sequence has been corrected herein. In that the amendments do not introduce new matter, their entry is respectfully requested.

B. Substitute Sequence Listing

A further substitute sequence listing is submitted herewith. Applicants have found that SEQ ID NO:4 in the previous sequence listings did not correspond to the HUV_HIII consensus sequence of Fig. 1B (see page 9, lines 1-2) and hence SEQ ID NO:4 in the attached substitute sequence listing has been corrected accordingly. Furthermore, SEQ ID NO:4 is hereby corrected on pages 8 and 15 of the application. In addition, separate sequence identifiers (SEQ ID NO's 27-30) have been given to the FR1-4 sequences in claim 41 added herein. In accordance with 37 C.F.R. §§1.821(f) and (g), the undersigned hereby states that the content of the paper and the computer readable sequence listings is the same. I further state that this submission includes no new matter.

C. Antibodies humanized according to the teachings of the instant application

As discussed in the interview, the consensus human variable domain of the instant claims has been used to humanize a number of antibodies, including:

1. *Anti-p185^{HER2} antibodies.* See Example 1 of the application, including Table 3 on page 72 (which describes humanized variants huMAb4D5-1-8) and page 65, lines 1-4 (concerning the use of a consensus human variable domain as recited in the claims herein). huMAb4D5-6 and huMAb4D5-8 had binding affinities which were suprisingly *superior* to that of the nonhuman antibody (muMAb4D5); see second to last column of Table 3. Repeated administration of the humanized anti-p185^{HER2} antibody huMAb4D5-8 has not lead to an immunogenic response in cancer patients treated therewith. See abstract of Baselga *et al.*, *J. Clin. Oncol.* 14(3):737-744 (1996), of record.

2. *Anti-CD3 antibodies.* See Example 3 on pages 79-88 of the application; and Fig. 5 as well as page 9, lines 25-31 concerning the use of a consensus human variable domain as claimed herein. [Note: In the Fig. 5 V_H consensus sequence (huIII), the last residue of FR2 is S, *i.e.* A→S, and eighth residue of FR3 is N, *i.e.* D→N, because of changes in 1987 to 1991 consensus sequence of Kabat *et al.*; such an equivalent consensus sequence and other changes in consensus sequences that result from the addition of further human antibody sequences to subsequent antibody compilations by Kabat *et al.* are clearly encompassed by the claims herein]. Humanized anti-CD3 variant (v1) was found to enhance the cytotoxic effects of activated human cytotoxic T lymphocytes (CTL) 4-fold against SK-BR-3 tumor cells overexpressing p185^{HER2} (page 81, lines 1-4). Variants of the humanized v1 antibody were made (v6 to v12; see page 82, line 22 and page 84, line 17 through to page 85, line 2 and page 86, lines 17-31), including the most potent variant, v9, which bound Jurkat cells almost as efficiently as the chimeric BsF(ab')$_2$ (page 86, lines 20-22).

3. *Anti-CD18 antibody.* See Example 4 on page 89 of the application and Figs. 6A and 6B with respect to a consensus human variable domain as claimed in the instant application. The binding affinity of the humanized anti-CD18 antibody (pH52-8.0/pH52-9.0; see Figs. 6A and 6B of

the application) was similar to the nonhuman H52 antibody; *i.e.* the humanized antibody has an affinity of 3.9 ± 0.9nM and murine H52 antibody has an affinity of 1.5 ± 0.3nM.

4. *Anti-IgE antibodies.* See Presta *et al. J. Immunol.* 151(5)2623-2632 (1993), of record. Use of a consensus human variable domain of the claims of the instant application is disclosed on page 2624 (column 1, first and third full paragraphs) and in Fig. 1. A number of humanized variants were made (see full paragraph 2 in column 1 on page 2624), including F(ab)-12 with only five framework region substitutions which exhibited binding comparable to the murine antibody (paragraph 2 on page 2631). Multidose administrations of full length anti-IgE variant 12 did not induce a human antihuman antibody response in allergic patients treated therewith (see column 1, last paragraph on page 311 of Shields *et al.*, *Int. Arch. Allergy Immunol.* 107:308-312 (1995), of record).

5. *Anti-CD11a antibodies.* See Werther *et al. J. Immnol.* 157:4986-4995 (1996), of record. Use of a consensus human variable domain as taught and claimed in the instant application is discussed in the first sentence of the Results section on page 4988 and in Fig. 1 (see note in paragraph 2 above, with respect to changes in 1987 to 1991 consensus sequences. Eight humanized variants were made (see Table 1 on page 4989), including HuIgG1 which had an apparent Kd similar to the parent murine antibody and comparable activity to the murine antibody in the cell adhesion and mixed leukocyte reaction (MLR) assays (see paragraph briging columns 1-2 on page 4993).

6. *Anti-VEGF antibodies.* See Presta *et al.* "Humanization of an anti-VEGF monoclonal antibody for the therapy of solid tumors and other disorders" *Cancer Research*, in press, pps. 1-32 of the manuscript, of record. The first paragraph on page 12 refers to the use of a consensus human variable domain as in the claims of this application. With respect to the consensus sequence in the figure on page 32 of the manuscript, see note in paragraph 2 above concerning change in 1987 to 1991 consensus sequences. As shown in Table 1 on page 29, twelve humanized anti-VEGF antibodies were made. The humanized antibody 12-IgG1 acquired the binding properties and biological activities of a high-affinity murine anti-VEGF MAb (see page 16,

last paragraph of this reference).

D. FR substitutions by Queen *et al.*

With respect to pending claim 10 herein reciting substitutions at specified sites in the V_H and V_L framework regions, as discussed at the interview, Queen *et al. PNAS, USA* 86:10029-10033 (1989) and US Patent 5,530,101 (the "101 patent") (cited by the office in the previous office action) use sequential numbering for the variable domain residues of the antibodies described in these references, whereas the claims of the instant application use Kabat numbering for the framework region residues (see page 14, lines 6-22 of the instant application). As requested by the Examiner in the interview, alignments of heavy chain variable domain (Exhibit A) and light chain variable domain (Exhibit B) sequences of the 101 patent (including the sequences for the murine and humanized anti-Tac antibody of Queen *et al.*) with sequential and Kabat residue numbering are attached. "murx" refers to the murine antibody sequence; "hzx" refers to the humanized antibody sequence; "H" is used for heavy chain variable domain sequences and "L" for light chain variable domain sequences. The sites at which the 101 patent refers to FR substitutions are:

Anti-Tac antibody (Figs. 1A and 1B of 101 patent)			
V_H FR substitions		**V_L FR substitutions**	
Sequential numbering	**Kabat numbering**	**Sequential numbering**	**Kabat numbering**
27H	27H	48L	48L
30H	30H	60L	60L
48H	48H	63L	63L
67H	66H		
68H	67H		
93H	89H		
95H	91H		
98H	94H		

107H	103H		
108H	104H		
109H	105H		
111H	107H		
Fd79 antibody (Figs. 2A and 2B of 101 patent)			
V_H FR substitions		**V_L FR substitutions**	
Sequential numbering	**Kabat numbering**	**Sequential numbering**	**Kabat numbering**
82H	81H	9L	9L
97H	93H	45L	41L
112H	103H	46L	42L
		53L	49L
		81L	77L
		83L	79L
Fd138-80 antibody (Figs. 3A and 3B of 101 patent)			
V_H FR substitions		**V_L FR substitutions**	
Sequential numbering	**Kabat numbering**	**Sequential numbering**	**Kabat numbering**
27H	27H	36L	36L
30H	30H	48L	48L
37H	37H	63L	63L
48H	48H	87L	87L
67H	66H		
68H	67H		
93H	89H		
98H	94H		

111H	103H		
112H	104H		
113H	105H		
115H	107H		
M195 antibody (Figs. 4A and 4B of the 101 patent)			
V_H FR substitions		**V_L FR substitutions**	
Sequential numbering	**Kabat numbering**	**Sequential numbering**	**Kabat numbering**
27H	27H	10L	10L
30H	30H	40L	36L
48H	48H	52L	48L
67H	66H	67L	63L
68H	67H	74L	70L
93H	89H	110L	106L
95H	91H		
98H	94H		
106H	103H		
107H	104H		
108H	105H		
110H	107H		
mik-β1 antibody (Figs. 5A and 5B of the 101 patent)			
V_H FR substitions		**V_L FR substitutions**	
Sequential numbering	**Kabat numbering**	**Sequential numbering**	**Kabat numbering**
1H	1H	13L	13L
29H	29H	41L	42L

30H	30H	70L	71L
49H	49H		
72H	72H		
73H	73H		
84H	82bH		
89H	86H		
90H	87H		
CMV5 antibody (Figs. 6A and 6B of the 101 patent)			
V_H FR substitions		**V_L FR substitutions**	
Sequential numbering	**Kabat numbering**	**Sequential numbering**	**Kabat numbering**
5H	5H	49L	49L
24H	24H		
27H	27H		
28H	28H		
30H	30H		
69H	68H		
80H	79H		
97H	93H		
AF2 antibody (Figs. 44A and 44B of the 101 patent)			
V_H FR substitions		**V_L FR substitutions**	
Sequential numbering	**Kabat numbering**	**Sequential numbering**	**Kabat numbering**
27H	27H	48L	48L
28H	28H	63L	63L
30H	30H	70L	70L

93H	89H		
95H	91H		
98H	94H		
107H	103H		
108H	104H		
109H	105H		
111H	107H		

Should the Examiner have any comments or questions concerning this amendment, he is invited to call Wendy Lee at (650) 225-1994 concerning these.

Respectfully submitted,
GENENTECH, INC.

Date: October 6, 1997 By: _____
Wendy M. Lee
Reg. No. 40,378

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-1994
Fax: (650) 952-9881

EXHIBIT A

Alignment of heavy chains from '101 patent

```
sequential 1        10        20        30        40        50
Kabat      1        10        20        30        40        50
           .        .         .         .         .         .
murxTacH   QVQLQQSGAELAKPGASVKWSCKASGYTFTSYRMHWVKQRPGQGLEWIGY
hzxTacH    QVQLVQSGAEVKKPGSSVKVSCKASGYTFTSYTMHWVRQAPGQGLEWIGY
EuH       QVQLVQSGAEVKKPGSSVKVSCKASGGTFSRSAIIWVRQAPGQGLEWMGG
murxMikH   QVQLKQSGPGLVQPSQSLSITCTVSGFSVTSYGVHWIRQSPGKGLEWLGV
hzxMikH    EVQLLESGGGLVQPGQSLRLSCAASGFTVTSYGVHWVRQAPGKGLEWVGV
LayH       AVQLLESGGGLVQPGGSLRLSCAASGFTFSASAMSWVRQAPGKGLEWVAW
murxAF2H   QVQLQQPGADLVMPGAPVKLSCLASGYIFTSSWINWVKQRPGRGLEWIGR
hzxAF2H    QVQLVQSGAEVKKPGSSVKVSCKASGYIFTSSWINWVRQAPGQGLEWMGR
murxCMV5H  EVQLQQSGPELVKPGASMKISCKASVYSFTGYTMNWVKQSHGQNLEWIGL
hzxCMV5H   QVQLVQSGAEVKKPGSSVRVSCKASGYSFTGYTMNWVRQAPGKGLEWVGL
murxFd138H QVQLQQSDAELVKPGASVKISCKVSGYTFTDHTIHWMKQRPEQGLEWFGY
hzxFd138H  QVQLVQSGAEVKKPGSSVKVSCKASGYTFTDHTIHWMRQAPGQGLEWFGY
murxFd79H  EMILVESGGGLVKPGASLKLSCAASGFTFSNYGLSWVRQTSDRRLEWVAS
hzxFd79H   EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYGLSWVRQAPGKGLEWVAS
murxM195H  EVQLQQSGPELVKPGASVKISCKASGYTFTDYNMHWVKQSHGKSLEWIGY
hzxM195H   QVQLVQSGAEVKKPGSSVKVSCKASGYTFTDYNMHWVRQAPGQGLEWIGY

sequential          60        70        80        90
Kabat         a     60        70        80   abc  90
                    .         .         .         .
murxTacH   INPSTGYTEYNQKFKDKATLTADKSSSTAYMQLSSLTFEDSAVYYCARG
hzxTacH    INPSTGYTEYNQKFKDKATITADESTNTAYMELSSLRSEDTAVYYCARG
EuH        IVPMFGPPNYAQKFQGRVTITADESTNTAYMELSSLRSEDTAFYFCAGG
murxMikH   IW-SGGSTDYNAAFISRLTISKDNSKSQVFFKVNSLQPADTAIYYCARA
hzxMikH    IW-SGGSTDYNAAFISRFTISRDNSKNTLYLQMNSLQAEDTAIYYCARA
LayH       KYENGNDKHYADSVNGRFTISRNDSKNTLYLQMNGLQAEVSAIYYCARD
murxAF2H   IDPSDGEVHYNQDFKDKATLTVDKSSSTAYIQLNSLTSEDSAVYYCARG
hzxAF2H    IDPSDGEVHYNQDFKDRVTITADESTNTAYMELSSLRSEDTAVYYCARG
murxCMV5H  INPYNGGTSYNQKFKGKATLYVDKSSNTAYMELLSLTSADSAVYYCTRR
hzxCMV5H   INPYNGGTSYNQKFKGRVTVSLKPSFNQAYMELSSLFSEDTAVYYCTRR
murxFd138H IYPRDGHTRYSEKFKGKATLTADKSASTAYMHLNSLTSEDSAVYFCARG
hzxFd138H  IYPRDGHTRYAEKFKGKATITADESTNTAYMELSSLRSEDTAVYFCARG
murxFd79H  ISRGGGRIYSPDNLKGRFTISKEDAKNTLYLQMSSLKSEDTALYYCLRE
hzxFd79H   ISRGGGRIYSPDNLKGRFTISRNDSKNTLYLQMNSLQAEDTALYYCLRE
murxM195H  IYPYNGGTGYNQKFKSKATLTVDNSSSTAYMDVRSLTSEDSAVYYCARG
hzxM195H   IYPYNGGTGYNQKFKSKATITADESTNTAYMELSSLRSEDTAVYYCARG
```

EXHIBIT A
(cont.)

```
sequential                  110
Kabat              103      110
                    •        •
murxTacH    GGV------FDYWGQGTTLTVSS
hzxTacH     GGV------FDYWGQGTLVTVSS
EuH         YGIYS----PEEYNGGLVTVSS
murxMikH    GDYNYDG--FAYWGQGTLVTVSA
hzxMikH     GDYNYDG--FAYWGQGTLVTVSS
LayH        AGPYVSPTFFAHWGQGTLVTVSS
murxAF2H    FLPW-----FADWGQGTLVTVSA
hzxAF2H     FLPW-----FADWGQGTLVTVSS
murxCMV5H   GFRDYS---MDYWGQGTSVTVSS
hzxCMV5H    GFRDYS---MDYWGQGTSVTVSS
murxFd138H  RDSRERNG-FAYWGQGTLVTVS-
hzxFd138H   RDSRERNG-FAYWGQGTLVTVSS
murxFd79H   GIYYADYGFFDVWGTGTTVIVSS
hzxFd79H    GIYYADYGFFDVWGQGTLVTVSS
murxM195H   RPA------MDYWGQGTSVTVSS
hzxM195H    RPA------MDYWGQGTLVTVSS
```

EXHIBIT B

Alignment of light chains from '101 patent

```
sequential  1        10        20        30              40
Kabat       1        10        20        30            40
            .        .         .         .             .
murxTacL    QIVLTQSPAIMSASPGEKVTITCSASSSIS-----YMHWFQQKPGTSPKL
hzxTacL     DIQMTQSPSTLSASVGDRVTITCSASSSIS-----YMHWYQQKPGKAPKL
EuL         DIQMTQSPSTLSASVGDRVTITCRASQSINT----WLAWYQQKPGKAPKL
murxMikL    QIVLTQSPAIMSASPGEKVTMTCSGSSSVS-----FMYWYQQRPGSSPRL
hzxMikL     DIQMTQSPSSLSASVGDRVTITCSGSSSVS-----FMYWYQQKPGKAPKL
LayL        DIQMTQSPSSLSVSVGDRVTITCQASQNVNA----YLNWYQQKPGLAPKL
murxAF2L    NIVMTQSPKSMYVSIGERVTLSCKASENVDT----YVSWYQQKPEQSPKL
hzxAF2L     DIQMTQSPSTLSASVGDRVTITCKASENVDT----YVSWYQQKPGKAPKL
murxCMV5L   DIVLTQSPATLSVTPGDSVSLSCRASQSISN----NLHWYQQKSHESPRL
hzxCMV5L    EIVLTQSPGTLSLSPGERATLSCRASQSISN----NLHWYQQKPGQAPRL
murxFd138L  DIVMTQSHKFMSTSVGDRVSITCKASQDVGS----AVVWHQQKSGQSPKL
hzxFd138L   DIQMTQSPSTLSASVGDRVTITCKASQDVGS----AVVWHQQKPGKAPKL
murxFd79L   DIVLTQSPASLAVSLGQRATISCRASQSVSTSTYNYMHWYQQKPGQPPKL
hzxFd79L    EIVMTQSPATLSVSPGERATLSCRASQSVSTSTYNYMHWYQQKPGQSPRL
murxM195L   DIVLTQSPASLAVSLGQRATISCRASESVDNYGISFMNWFQQKPGQPPKL
hzxM195L    DIQMTQSPSSLSASVGDRVTITCRASESVDNYGISFMNWFQQKPGKAPKL

sequential    50        60        70        80        90
Kabat         50        60        70        80        90
              .         .         .         .         .
murxTacL    WIYTTSNLASGVPARFSGSGSGTSYSLTISRMEAEDAATYYCHQRSTYPL
hzxTacL     LIYTTSNLASGVPARFSGSGSGTEFTLTISSLQPDDFATYYCHQRSTYPL
EuL         LMYKASSLESGVPSRFIGSGSGTEFTLTISSLQPDDFATYYCQQYNSDSK
murxMikL    LIYDTSNLASGVPVRFSGSGSGTSYSLTISRMEAEDAATYYCQQWSTYPL
hzxMikL     LIYDTSNLASGVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQWSTYPL
LayL        LIYGASTREAGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQYNNWPP
murxAF2L    LIYGASNRYTGVHDRFTGSGSATDFTLTISSVQAEDLADYHCGQSYNYPF
hzxAF2L     LIYGASNRYTGVPSRFSGSGSGTDFTLTISSLQPDDFATYYCGQSYNYPF
murxCMV5L   LIKYASQSISGIPSRFSGSGSGTDFTLSVNGVETEDFGMYFCQQSNSWPH
hzxCMV5L    LIKYASQSISGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSNSWPH
murxFd138L  LIYWASTRHTGVPDRFTGSGSGTDFTLTITNVQSEDLADYFCQQYSIFPL
hzxFd138L   LIYWASTRHTGVPSRFTGSGSGTEFTLTISSLQPDDFATYFCQQYSIFPL
murxFd79L   LIKYASNLESGVPARFSGSGFGTDFTLNIHPVEEEDTVTYYCQHSWEIPY
hzxFd79L    LIKYASNLESGIPARFSGSGSGTEFTLTISRLESEDFAVYYCQHSWEIPY
murxM195L   LIYAASNQGSGVPARFSGSGSGTDFSLNIHPMEEDDTAMYFCQQSKEVPW
hzxM195L    LIYAASNQGSGVPSRFSGSGSGTDFTLNISSLQPDDFATYYCQQSKEVPW
```

EXHIBIT B
(cont.)

```
sequential    100
Kabat         100
                •
murxTacL    TFGSGTKLELK
hzxTacL     TFGQGTKVEVK
EuL         MFGQGTKVEVK
murxMikL    TFGAGTKLELK
hzxMikL     TFGQSTKVEVK
LayL        TFGQGTKVEVK
murxAF2L    TFGSGTKLEIK
hzxAF2L     TFGQGTKVEVK
murxCMV5L   TFGGGTKLEIK
hzxCMV5L    TFGQGTKVEIK
murxFd138L  TFGAGTRLELK
hzxFd138L   TFGQGTKVEVK
murxFd79L   TFGGGTKLEIK
hzxFd79L    TFGQGTRVEIK
murxM195L   TFGGGTKLEIK
hzxM195L    TFGQGTKVEIK
```

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Carter, Paul J.
Presta, Leonard G.

(ii) TITLE OF INVENTION: Method for Making Humanized Antibodies

(iii) NUMBER OF SEQUENCES: 30

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/146206
(B) FILING DATE: 17-Nov-1993
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 07/715272
(B) FILING DATE: 14-JUN-1991

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lee, Wendy M.
(B) REGISTRATION NUMBER: 40,378
(C) REFERENCE/DOCKET NUMBER: P0709P1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650/225-1994
(B) TELEFAX: 650/952-9881

(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                 100                 105

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

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 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                 100                 105

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                 100                 105

Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                 110                 115                 120
```

(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                 100                 105

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                 100                 105

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

(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 GCHSCDCCGA 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:

(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                  100                 105

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
        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                  100                 105
```

6

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

(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:

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                 100                 105
```

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

Ser Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                95                 100                 105

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                 100                 105

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                 100                 105

Ser Gly Leu Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
                 110                 115                 120

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                 100                 105

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

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
95 100 105

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

Val Val Thr Val Thr Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr
215 220 225

12

```
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

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                 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
        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:

```
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                 100                 105

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

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
```

15

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
95 100 105

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:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

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
20 25

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala
1 5 10 14

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Asn Thr Leu Tyr Leu
1 5 10 15

Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
20 25 30

Ala Arg
32

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
1 5 10 11

11/6/97

This Raw Listing contains the General Information Section and up to the first 5 pages.

ENTERED

SEQUENCE LISTING

(1) General Information:

(i) APPLICANT: Carter, Paul J.
Presta, Leonard G.

(ii) TITLE OF INVENTION: Method for Making Humanized Antibodies

(iii) NUMBER OF SEQUENCES: 26

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
--> OK **(A) APPLICATION NUMBER: 08/146206**
(B) FILING DATE: 17-Nov-1993
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 07/715272
(B) FILING DATE: 14-JUN-1991

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lee, Wendy M.
(B) REGISTRATION NUMBER: 40,378
(C) REFERENCE/DOCKET NUMBER: P0709P1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650/225-1994
(B) TELEFAX: 650/952-9881

(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                 100                 105

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
```

```
100   Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
101                    80                  85                  90
102
103   Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
104                    95                 100                 105
105
106   Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
107                   110                 115                 120
108
109  (2) INFORMATION FOR SEQ ID NO:3:
110
111     (i) SEQUENCE CHARACTERISTICS:
112         (A) LENGTH: 109 amino acids
113         (B) TYPE: Amino Acid
114         (D) TOPOLOGY: Linear
115
116      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
117
118   Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
119     1               5                  10                  15
120
121   Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Ser
122                    20                  25                  30
123
124   Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
125                    35                  40                  45
126
127   Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
128                    50                  55                  60
129
130   Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
131                    65                  70                  75
132
133   Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
134                    80                  85                  90
135
136   Tyr Asn Ser Leu Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
137                    95                 100                 105
138
139   Ile Lys Arg Thr
140                109
141
142  (2) INFORMATION FOR SEQ ID NO:4:
143
144     (i) SEQUENCE CHARACTERISTICS:
145         (A) LENGTH: 120 amino acids
146         (B) TYPE: Amino Acid
147         (D) TOPOLOGY: Linear
148
149      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
150
151   Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
152     1               5                  10                  15
```

153
154 Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
155 20 25 30
156
157 Asp Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
158 35 40 45
159
160 Glu Trp Val Ala Val Ile Ser Glu Asn Gly Gly Tyr Thr Arg Tyr
161 50 55 60
162
163 Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
164 65 70 75
165
166 Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
167 80 85 90
168
169 Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
170 95 100 105
171
172 Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
173 110 115 120
174
175 (2) INFORMATION FOR SEQ ID NO:5:
176
177 (i) SEQUENCE CHARACTERISTICS:
178 (A) LENGTH: 109 amino acids
179 (B) TYPE: Amino Acid
180 (D) TOPOLOGY: Linear
181
182 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
183
184 Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val
185 1 5 10 15
186
187 Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Asn
188 20 25 30
189
190 Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly His Ser Pro Lys
191 35 40 45
192
193 Leu Leu Ile Tyr Ser Ala Ser Phe Arg Tyr Thr Gly Val Pro Asp
194 50 55 60
195
196 Arg Phe Thr Gly Asn Arg Ser Gly Thr Asp Phe Thr Phe Thr Ile
197 65 70 75
198
199 Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
200 80 85 90
201
202 His Tyr Thr Thr Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu
203 95 100 105
204
205 Ile Lys Arg Ala

206 109

207

208 (2) INFORMATION FOR SEQ ID NO:6:

209

210 (i) SEQUENCE CHARACTERISTICS:
211 (A) LENGTH: 120 amino acids
212 (B) TYPE: Amino Acid
213 (D) TOPOLOGY: Linear

214

215 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

216

217 Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
218 1 5 10 15

219

220 Ala Ser Leu Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
221 20 25 30

222

223 Asp Thr Tyr Ile His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu
224 35 40 45

225

226 Glu Trp Ile Gly Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
227 50 55 60

228

229 Asp Pro Lys Phe Gln Asp Lys Ala Thr Ile Thr Ala Asp Thr Ser
230 65 70 75

231

232 Ser Asn Thr Ala Tyr Leu Gln Val Ser Arg Leu Thr Ser Glu Asp
233 80 85 90

234

235 Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
236 95 100 105

237

238 Ala Met Asp Tyr Trp Gly Gln Gly Ala Ser Val Thr Val Ser Ser
239 110 115 120

240

241 (2) INFORMATION FOR SEQ ID NO:7:

242

243 (i) SEQUENCE CHARACTERISTICS:
244 (A) LENGTH: 27 base pairs
245 (B) TYPE: Nucleic Acid
246 (C) STRANDEDNESS: Single
247 (D) TOPOLOGY: Linear

248

249 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

250

251

252 TCCGATATCC AGCTGACCCA GTCTCCA 27

253

254 (2) INFORMATION FOR SEQ ID NO:8:

255

256 (i) SEQUENCE CHARACTERISTICS:
257 (A) LENGTH: 31 base pairs
258 (B) TYPE: Nucleic Acid

SEQUENCE VERIFICATION REPORT
PATENT APPLICATION *US/08/146,206B*

INPUT SET: S20851.raw

Line	Error	Original Text
27	Wrong application Serial Number	(A) APPLICATION NUMBER: 08/146206

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/146,206	11/17/93	CARTER P	789P1

18M1/1223

JANET E. HASAK
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO CA 94080-4990

EXAMINER	
NOLAN, P	35
ART UNIT	PAPER NUMBER
1816	

DATE MAILED: 12/23/97

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Office Action Summary	Application No. 08/146,206	Applicant(s) Carter et al.
	Examiner Patrick J. Nolan	Group Art Unit 1816

☒ Responsive to communication(s) filed on *6-27-97, 9-1-97 and 10-7-97*.

☒ This action is **FINAL.**

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire *3* month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *1-8, 10-12, 15, and 22-42* is/are pending in the application.

Of the above, claim(s) _____ is/are withdrawn from consideration.

☐ Claim(s) _____ is/are allowed.

☒ Claim(s) *1-8, 10-12, 15, and 22-41* is/are rejected.

☒ Claim(s) *42* is/are objected to.

☐ Claims _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐approved ☐disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☒ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

--- SEE OFFICE ACTION ON THE FOLLOWING PAGES ---

Serial No. 08/146,206

Art Unit 1816

1. Claims 1-8, 10-12, 15 and 22-42 are pending.

Double Patenting

2. The non-statutory double patenting rejection, whether of the obviousness-type or non-obviousness-type, is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent. *In re Thorington,* 418 F.2d 528, 163 USPQ 644 (CCPA 1969); *In re Vogel,* 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Van Ornum,* 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Longi,* 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); and *In re Goodman,* 29 USPQ2d 2010 (Fed. Cir. 1993).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(b) and (c) may be used to overcome an actual or provisional rejection based on a non-statutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.78(d). Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

3. Claims 1-12, 15 stand 19-25 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-12, 15 and 19 of copending application Serial No. 08/439,004.

Applicant's request these rejection be held in abeyance until the prosecution of the two pending cases are completed.

Claim Rejections - 35 USC § 102

4. The following is a quotation of the appropriate paragraphs of 35 U.S.C. § 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless --
>
> e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section

371(c) of this title before the invention thereof by the applicant for patent.

5. Claims 1-8, 10-12, 15 and 22-24 stand rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent 5,530,101 (82).

Applicant's arguments filed 6-23-97 have been fully considered but are not found persuasive.

6. Applicant argues that the '101 patent does not teach the determination of residues which will disrupt the V_L-V_H interface as part of their method to make a humanized antibody.

However, Applicant's claims are drawn to using one of the following effects recited in claim 1 and 23, part (f), not all three.

7. Applicant argues that the determination of residues being exposed to the CDR region is not the same as the '101 teaching of whether the residue "interacts with a CDR".

Protein chemistry dictates that for an amino acid residue to interact with another amino acid residues it needs to be exposed to it.

8. Applicant argues that since the '101 patent does not specifically teach glycosylation of the residue being a factor for selection it cannot be used as a prior art reference.

The teaching of glycosylation effects on amino acid residues, is of record, as taught by Roitt et al., submitted in the last office action. Roitt is an educational textbook demonstrating concepts well known to those in the art.

9. Applicant argues that claims drawn to specific residue changes have been amended to distinguish the claims from the '101 patent. Applicant has also demonstrated the numbering difference between the '101 patent and the current application.

If applicant wishes to distinguish over the prior art, they may do so by claiming the actual numbering system used in the actual claim.

The following new grounds of rejections are necessitated by the amendments filed 6-27-97, 9-1-97 and 10-7-97.

10. Claims 22-25, 38, and 39 are rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent 5,693,762 (A).

The '762 patent teaches the aligning of heavy chain immunoglobulin regions for the creation of a consensus sequence to be used in making a humanized antibody (column 13, lines 4-26 and claims 7-9 and 20, in particular). The '762 patent also teaches that in selecting which consensus framework sequence to be used, the acceptor immunoglobulin most likely should be as homologous to the donor sequence as possible (i.e. same isotype) (column 13).

The prior art teachings anticipate the claimed invention.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. § 103 which forms the basis for all obviousness rejections set forth in this Office action:

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(f) or (g) prior art under 35 U.S.C. 103(a).

11. Claims 26-36 and 40-41 are rejected under 35 U.S.C. § 103 as being unpatentable over U.S. Patent 5,693,762 (A), in view of Kabat et al.

The `762 patent has been discussed supra. The claimed

invention differs from the prior art teachings only by the recitation the Ig gamma isotype sequences used to make a consensus heavy chain framework region.

However, Kabat et al., teach the sequences of all known Ig gamma subtypes.

Therefore it would have been prima facie obvious to one of skill in the art at the time the invention was made to use the teachings of the '762 patent and align all of the known Ig gamma heavy chains for the creation of a consensus sequence with the expectation that said consensus sequence immunoglobulin would have a smaller chance of changing the an amino acid near the CDR's that distorts their conformation, as taught by the '762 patent (column 13).

12. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL.** See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for response to this final action is set to expire THREE MONTHS from the date of this action. In the event a first response is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event will the statutory period for response expire later than SIX MONTHS from the date of this final action.

13. If attempts to reach the examiner are unsuccessful, the examiner's supervisor, Christina Chan, can be reached at (703) 305-3973. The FAX number for our group, 1816, is (703) 305-7939. Any inquiry of a general nature relating to the status of this application or proceeding should be directed to the Group receptionist, whose telephone number is (703) 308-0196.

Patrick J. Nolan, Ph.D.
December 19, 1997

F.C. Eisenschenk
Primary Examiner
December 19, 1997

Notice of References Cited

Application No.	Applicant(s)		
08/146,206	Carter et al.		
Examiner	Group Art Unit	Page 1 of 1	
Patrick J. Nolan	1816		

U.S. PATENT DOCUMENTS

		DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS
	A	5,693,762	12-2-97	Queen et al.	530	387.2
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS
	N						
	O						
	P						
	Q						
	R						
	S						
	T						

NON-PATENT DOCUMENTS

		DOCUMENT (Including Author, Title, Source, and Pertinent Pages)	DATE
	U		
	V		
	W		
	X		

12/05/01

Gp 1644

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1644 Examiner: P. Nolan RECEIVED APR 16 1998 CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on April 8, 1998 Nicole Kehoe

NOTICE OF CHANGE OF ADDRESS AND AREA CODE

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Please direct all future communications in connection with the above referenced patent application to:

Genentech, Inc.
1 DNA Way
South San Francisco, CA 94080-4990

Please also note the change in area code from 415 to 650 (see below).

Respectfully submitted,
GENENTECH, INC.

Date: April 7, 1998

By: _____
Wendy M. Lee
Reg. No. 40,378

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-1994
Fax: (650) 952-9881

GP1644

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1644 Examiner: P. Nolan CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on June 23, 1998 Yvonne E. Carter

NOTICE OF APPEAL

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicant hereby appeals to the Board of Appeals and Interferences from the decision dated 23 December 1997, of the Primary Examiner finally rejecting claims 1-8, 10-12, 15, and 22-41 and objecting to claim 42.

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $310 to cover the fees for this appeal and to charge the deposit account for any further fees in regard to this patent application. **A duplicate copy of this Notice is enclosed for this purpose.**

07/01/1998 SSAHDARA 00000105 070630 08146206
01 FC:119 310.00 CH

Respectfully submitted,
GENENTECH, INC.

Date: June 23, 1998

By: _____
Richard B. Love
Reg. No. 34,659

RECEIVED
JUL 6 1998
GROUP 1600

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-1994
Fax: (650) 952-9881

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1644 Examiner: P. Nolan CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on June 23, 1998 Yvonne E. Carter

PETITION AND FEE FOR THREE MONTH EXTENSION OF TIME (37 CFR 1.136(a))

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicant petitions the Commissioner of Patents and Trademarks to extend the time for response to the FINAL OFFICE ACTION dated 23 December 1997 for three month(s) from 23 March 1998 to 23 June 1998. The extended time for response does not exceed the statutory period.

Please charge Deposit Account No. 07-0630 in the amount of $950.00 to cover the cost of the extension. Any deficiency or overpayment should be charged or credited to this deposit account. A duplicate of this sheet is enclosed.

07/01/1998 SSANDARA 00000105 070630 08146206
02 FC:117 950.00 CH

Respectfully submitted,
GENENTECH, INC.

Date: June 23, 1998

By: Richard B. Love
Richard B. Love
Reg. No. 34,659

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-1994
Fax: (650) 952-9881

#37

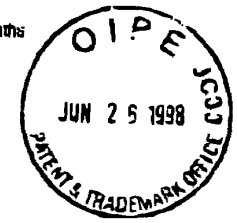
In re Application of Paul J. Carter et al.
Serial No.: 08/146,206
Filed On: November 17, 1993
Mailed On: 23 June 1998

Docket No.: P0709P1
By: Richard B. Love
Reg. No.: 34,659

LS

The following has been received in the U.S. Patent Office on the date stamped:

- X Petition to Extent Time for Three Months
- X Notice of Appeal Transmittal
- X Fees $ 1,260.00
- X Postcard



UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE OFFICE OF ENROLLMENT AND DISCIPLINE

LIMITED RECOGNITION UNDER 37 CFR § 10.9(b)

Wendy M. Lee is hereby given limited recognition under 37 CFR § 10.9(b) as an employee of Genentech, Inc. to prepare and prosecute patent applications and to represent patent applicants wherein Genentech, Inc. is the assigneed of record of the entire interest. This limited recognition shall expire on the date appearing below, or when whichever of the following events first occurs prior to the date appearing below: (i) Wendy M. Lee ceases to lawfully reside in the United States, (ii) Wendy M. Lee's employment with Genentech, Inc. ceases or is terminated, or (iii) if Wendy M. Lee ceases to remain or reside in the United States on a H-1 visa.

This document constitutes proof of such recognition. The original of this document is on file in the Office of Enrollment and Discipline of the U.S. Patent and Trademark Office.

EXPIRES: DECEMBER 9, 1995

Cameron Weiffenbach, Director
Office of Enrollment and Discipline

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1644 Examiner: P. Nolan CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on June 23, 1998 Yvonne E. Carter

#38 Supp 8-17-98 FORMAL PLEASE ENTER P.N. 8-13-98

PETITION AND FEE FOR THREE MONTH EXTENSION OF TIME
(37 CFR 1.136(a))

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicant petitions the Commissioner of Patents and Trademarks to extend the time for response to the FINAL OFFICE ACTION dated 23 December 1997 for three month(s) from 23 March 1998 to 23 June 1998. The extended time for response does not exceed the statutory period.

Please charge Deposit Account No. 07-0630 in the amount of $950.00 to cover the cost of the extension. Any deficiency or overpayment should be charged or credited to this deposit account. A duplicate of this sheet is enclosed.

08/19/1998 DLYONS 00000007 070630 08146206
01 FC:117 950.00 CH
02 FC:119 310.00 CH

Respectfully submitted,
GENENTECH, INC.

Date: June 23, 1998

By: _____
Richard B. Love
Reg. No. 34,659

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-1994
Fax: (650) 952-9881

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1644 Examiner: P. Nolan CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on June 23, 1998 Yvonne E. Carter

NOTICE OF APPEAL

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicant hereby appeals to the Board of Appeals and Interferences from the decision dated 23 December 1997, of the Primary Examiner finally rejecting claims 1-8, 10-12, 15, and 22-41 and objecting to claim 42.

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $310 to cover the fees for this appeal and to charge the deposit account for any further fees in regard to this patent application. A duplicate copy of this Notice is enclosed for this purpose.

Respectfully submitted,
GENENTECH, INC.

Date: June 23, 1998

By: _____
Richard B. Love
Reg. No. 34,659

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-1994
Fax: (650) 952-9881

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

08/146,206

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKETT NO.

EXAMINER

ART UNIT	PAPER NUMBER
	37

DATE MAILED:

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) Wendy Lee (3) _____

(2) Patrick Nolan (4) _____

Date of interview 8-13-98

Type: ☒ Telephonic ☒ Personal (copy is given to ☐ applicant ☒ applicant's representative).

Exhibit shown or demonstration conducted: ☒ Yes ☐ No. If yes, brief description: Wall street Journal article

Agreement ☐ was reached with respect to some or all of the claims in question. ☒ was not reached.

Claims discussed: Newly Proposed Claims Faxed 8-10-98

Identification of prior art discussed: Green Patent 5,693,762

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: Discussed unexpected results to overcome 103 rejections

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

☒ 1. It is not necessary for applicant to provide a separate record of the substance of the interview.

Unless the paragraph below has been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1-7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the interview.

☐ 2. Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action. Applicant is not relieved from providing a separate record of the substance of the interview unless box 1 above is also checked.

Patrick J. Nolan
Examiner's Signature

PTOL-413 (REV. 2 -93)

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AF / Gpu 1644

Patent Docket P0709P10 ⌀

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1644 Examiner: P. Nolan RECEIVED SEP 0 1 1998 GROUP 1800 **CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on August 24, 1996 Wendy M. Lee

AMENDMENT TRANSMITTAL

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Transmitted herewith is an Amendment under 37 C.F.R. §1.129(a) in the above-identified application.

The fee has been calculated as shown below.

	Claims Remaining After Amendment		Highest No. Previously Paid For	Present Extra	Rate	Additional Fees
Total	72	-	35	37	x 22 =	$814.00
Independent	7	-	10	0	x 78 =	$0.00
__ First Presentation of Multiple Dependent Claims					+ 250 =	
					Total Fee Calculation	$814.00

X Amendment under 37 C.F.R. §1.129(a) submitted with fee of $750.00 pursuant to 37 C.F.R. §1.17(r)

X The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $1,564.00 pursuant to 37 C.F.R. §1.17(r). **A duplicate copy of this transmittal is enclosed.**

X A Declaration of Steven Shak with Exhibits A-F is enclosed.

X A Supplemental Information Disclosure Statement, PTO-1449 Form, and copies of Refs. 218-224 are enclosed.

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR 1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. **A duplicate copy of this sheet is enclosed.**

Respectfully submitted,
GENENTECH, INC.

Date: August 24, 1998

By: _____
Wendy M. Lee
Reg. No.40,378

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

Revised (10/13/95)

#39 Amdt (Rule 116) Claims 08/03/98

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1644 Examiner: P. Nolan CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on August 24, 1998 Wendy M. Lee

AMENDMENT UNDER 37 C.F.R. §1.129(a)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

This paper is being filed in response to the Office Action mailed December 23, 1997. In the Office Action, the Examiner issued a final rejection of claims 1-8, 10-12, 15 and 22-41 and objected to claim 42. Applicants filed a Notice of Appeal on June 23, 1998. Applicants have not yet filed an Appeal Brief. Accordingly, the present response is being submitted under Section 1.129(a) along with the fee set forth in Section 1.17(r). In that August 23, 1998 fell on a Sunday, this amendment is timely filed.

Entry of the following amendment is respectfully requested:

IN THE CLAIMS:

Please cancel claims 1-8, 10-12, 15 and 22-42 without prejudice or disclaimer of the subject matter claimed therein.

H

Please add the following claims:

--43. (New) A humanized antibody variable domain comprising a non-human Complementarity Determining Region (CDR) incorporated into a human antibody variable domain, and further comprising an 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.

44. ~~(New)~~ The humanized variable domain of claim 43 wherein the substituted residue is ~~the residue~~ found at the corresponding location of the non-human antibody from which ~~the non-human~~ CDR was obtained.

~~3~~ 45. (New) The humanized variable domain of claim ~~43~~ 1 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

~~4~~ 46. (New) The humanized variable domain of claim ~~43~~ 11 wherein the human antibody variable domain is a consensus human variable domain.

~~5~~ 47. (New) The humanized variable domain of claim ~~43~~ 1 wherein the residue at site 4L has been substituted.

~~6~~ 48. (New) The humanized variable domain of claim ~~43~~ 1 wherein the residue at site 38L has been substituted.

~~7~~ 49. (New) The humanized variable domain of claim ~~43~~ 1 wherein the residue at site 43L has been substituted.

~~8~~ 50. (New) The humanized variable domain of claim ~~43~~ 1 wherein the

93

residue at site 44L has been substituted.

51. (New) The humanized variable domain of claim 43 wherein the residue at site 58L has been substituted.

52. (New) The humanized variable domain of claim 43 wherein the residue at site 62L has been substituted.

53. (New) The humanized variable domain of claim 43 wherein the residue at site 65L has been substituted.

54. (New) The humanized variable domain of claim 43 wherein the residue at site 66L has been substituted.

55. (New) The humanized variable domain of claim 43 wherein the residue at site 67L has been substituted.

56. (New) The humanized variable domain of claim 43 wherein the residue at site 68L has been substituted.

57. (New) The humanized variable domain of claim 43 wherein the residue at site 69L has been substituted.

58. (New) The humanized variable domain of claim 43 wherein the residue at site 73L has been substituted.

59. (New) The humanized variable domain of claim 43 wherein the residue at site 85L has been substituted.

60. (New) The humanized variable domain of claim 43 wherein the residue at site 98L has been substituted.

61. (New) The humanized variable domain of claim 43 wherein the

residue at site 2H has been substituted.

62. (New) The humanized variable domain of claim 43 wherein the residue at site 4H has been substituted.

63. (New) The humanized variable domain of claim 43 wherein the residue at site 36H has been substituted.

64. (New) The humanized variable domain of claim 43 wherein the residue at site 39H has been substituted.

65. (New) The humanized variable domain of claim 43 wherein the residue at site 43H has been substituted.

66. (New) The humanized variable domain of claim 43 wherein the residue at site 45H has been substituted.

67. (New) The humanized variable domain of claim 43 wherein the residue at site 69H has been substituted.

68. (New) The humanized variable domain of claim 43 wherein the residue at site 70H has been substituted.

69. (New) The humanized variable domain of claim 43 wherein the residue at site 74H has been substituted.

70. (New) The humanized variable domain of claim 43 wherein the residue at site 92H has been substituted.

71. (New) An antibody comprising the humanized variable domain of claim 43.

72. (New) An antibody which binds $p185^{HER2}$ and comprises a

humanized antibody variable domain comprising a non-human Complementarity Determining Region (CDR) 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.

73. ~~(New)~~ The antibody of claim 72 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR was obtained.

74. (New) The antibody of claim 72 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

75. (New) The antibody of claim 72 wherein the human antibody variable domain is a consensus human variable domain.

76. (New) The antibody of claim 72 wherein the residue at site 4L has been substituted.

77. (New) The antibody of claim 72 wherein the residue at site 38L has been substituted.

78. (New) The antibody of claim 72 wherein the residue at site 43L has been substituted.

79. (New) The antibody of claim 72 wherein the residue at site 44L has been substituted.

80. (New) The antibody of claim 72 wherein the residue at site 46L has been substituted.

81. (New) The antibody of claim 72 wherein the residue at site 58L has been substituted.

82. (New) The antibody of claim 72 wherein the residue at site 62L has been substituted.

83. (New) The antibody of claim 72 wherein the residue at site 65L has been substituted.

84. (New) The antibody of claim 72 wherein the residue at site 66L has been substituted.

85. (New) The antibody of claim 72 wherein the residue at site 67L has been substituted.

86. (New) The antibody of claim 72 wherein the residue at site 68L has been substituted.

87. (New) The antibody of claim 72 wherein the residue at site 69L has been substituted.

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88. (New) The antibody of claim 72 wherein the residue at site 73L has been substituted.

89. (New) The antibody of claim 72 wherein the residue at site 85L has been substituted.

90. (New) The antibody of claim 72 wherein the residue at site 98L has been substituted.

91. (New) The antibody of claim 72 wherein the residue at site 2H has been substituted.

92. (New) The antibody of claim 72 wherein the residue at site 4H has been substituted.

93. (New) The antibody of claim 72 wherein the residue at site 36H has been substituted.

94. (New) The antibody of claim 72 wherein the residue at site 39H has been substituted.

95. (New) The antibody of claim 72 wherein the residue at site 43H has been substituted.

96. (New) The antibody of claim 72 wherein the residue at site 45H has been substituted.

97. (New) The antibody of claim 72 wherein the residue at site 69H has been substituted.

98. (New) The antibody of claim 72 wherein the residue at site 70H has been substituted.

H1 cont'd

99. (New) The antibody of claim 72 wherein the residue at site 74H has been substituted.

100. (New) The antibody of claim 72 wherein the residue at site 75H has been substituted.

101. (New) The antibody of claim 72 wherein the residue at site 76H has been substituted.

102. (New) The antibody of claim 72 wherein the residue at site 78H has been substituted.

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103. (New) The antibody of claim 72 wherein the residue at site 92H has been substituted.

104. (New) A humanized antibody variable domain comprising a non-human Complementarity Determining Region (CDR) 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.

105. (New) An antibody which lacks significant immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient and comprises (a) non-human Complementarity Determining Region (CDR) 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.

106. (New) An antibody which lacks significant immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient and comprises a consensus human variable domain 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 comprising a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) comprises a glycosylation site which affects the antigen binding or affinity of the antibody; or (d) participates in the V_L-V_H interface by

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affecting the proximity or orientation of the V_L and V_H regions with respect to one another.

107. (New) The antibody of claim 106 comprising a non-human FR residue which noncovalently binds antigen directly.

108. (New) The antibody of claim 106 comprising a non-human FR residue which interacts with a CDR.

109. (New) The antibody of claim 106 comprising a non-human FR residue which comprises a glycosylation site which affects the antigen binding or affinity of the antibody.

110. (New) The antibody of claim 106 comprising a non-human FR residue which 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.

111. (New) A humanized antibody comprising a consensus human variable domain of human V_H subgroup III, wherein amino acid residues forming Complementarity Determining Regions (CDRs) thereof comprise non-human antibody amino acid residues, and further comprising a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) comprises 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.

112. (New) The humanized antibody of claim 111 which lacks significant immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient.

113. (New) A humanized variant of a non-human parent antibody which binds an antigen with better affinity than the parent antibody and comprises a consensus human variable domain 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 comprising a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) comprises 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.

114. (New) The humanized variant of claim 113 which binds the antigen at least about 3-fold more tightly than the parent antibody.--

REMARKS

The undersigned confirms having met with Examiner Nolan in the personal interview on August 13, 1998 and thanks the Examiner for the courtesies extended in the interview. In the interview, the undersigned pointed out that claim 42 was not rejected, but was objected-to in the above-noted final Office Action. However, the basis for the objection was not elaborated in the body of the Office Action. The Examiner indicated that claim 42 was objected to for depending on a rejected claim (*i.e.* claim 22). Other issues discussed in the interview will be mentioned herein-below where appropriate.

Amendments

The previously pending claims are cancelled herein without prejudice and without disclaimer of the subject matter claimed

therein and without acquiescing in any rejection or objection raised by the Office. Applicants reserve the right to pursue continuing application(s) directed to cancelled claims. The claims herein correspond to those discussed in the interview and are believed to be allowable.

Former claim/specification basis for each of the claims added herein can be found at least as follows:

Claims 43 and 47-70 - claim 10 as amended 10-7-97; and page 6, lines 21-22 for "utilizing the numbering system set forth in Kabat"
Claim 44 - original claim 11
Claim 45 - original claim 12
Claim 46 - language from claim 1
Claim 71 - page 11, lines 3-4
Claims 72 and 76-103 - claim 10 as amended 10-7-97; page 63, line 21 for "antibody which binds $p185^{HER2}$"; and page 6, lines 21-22 for "utilizing the numbering system set forth in Kabat"
Claim 73 - original claim 11
Claim 74 - original claim 12
Claim 75 - language from claim 1
Claim 104 - claim 10 as amended 10-7-97; claim 1 for "consensus human variable domain"; and page 6, lines 21-22 for "utilizing the numbering system set forth in Kabat"
Claim 105 - claims 10 and 42 from the amendment 10-7-97; and page 6, lines 21-22 for "utilizing the numbering system set forth in Kabat"
Claim 106 - combination of claims 22, 23 and 42
Claims 107-110 - claim 23
Claim 111 - combination of claims 22, 23 and 26
Claim 112 - claim 42
Claim 113 - claims 22 and 23; page 71, lines 1-2 and Table 3 on

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page 72 showing humanized variants with improved binding affinity compared to the murine parent antibody.
Claim 114 - page 71, lines 1-2

In that the claims do not introduce new matter, their entry is respectfully requested.

Information Disclosure

1. In the above-mentioned interview, the undersigned inquired as to the status of the IDS carried to the PTO September 1997 citing references 100-207. The Examiner indicated he had this IDS and the references and would consider them with respect to the above application. Applicants await receipt of a copy of the initialed PTO-1449 form indicating consideration of the cited art.

2. A further supplemental IDS is submitted herewith. Applicants respectfully request consideration of the art cited in this supplemental IDS with respect to the instant application.

Provisional Double Patenting Rejection

Claims 1-12, 15 and 19-25 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-12, 15 and 19 of copending application Serial No. 08/439,004. This rejection is moot as USSN 08/439,004 is now abandoned.

Section 102(e) - US Patent 5,530,101

Claims 1-8, 10-12, 15 and 22-24 are rejected under 35 USC §102(e) as being anticipated by US Patent 5,530,101 ("the '101 patent")

With respect to claim 10, the Examiner states in item 9 of the Office Action that the claim may be distinguished over the prior art by claiming the actual numbering system used in the actual

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claim. In order to expedite prosecution, Applicants have followed the Examiner's suggestion and recite the numbering system of Kabat in independent claims 43, 72, 104 and 105 herein for claim precision.

Further patentable features in these claims and the claims which depend thereon include, without limitation: the target antigen $p185^{HER2}$ in claim 72 (which is not taught in the '101 patent); a consensus human variable domain which, as will be explained below, is not taught or enabled by the '101 patent; and the antibody which lacks significant immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient (see comments below).

Applicants submit that independent claims 43, 72, 104 and 105 herein as well as the claims which depend thereon are patentable over the cited art.

Reconsideration and withdrawal of the Section 102 rejection is respectfully requested.

Section 102(e) - US Patent 5,693,762

Claims 22-25, 38 and 39 are rejected under 35 USC §102(e) as being anticipated by US Patent 5,693,762 ("the '762 patent").

The Examiner asserts that the '762 patent taught the aligning of heavy chain immunoglobulin regions for the creation of a consensus sequence to be used in making a humanized antibody and that the acceptor immunoglobulin most likely should be as homologous to the donor sequence as possible (*i.e.* same isotype).

Applicants submit that the '762 patent does not anticipate the instant invention.

Importantly, the '762 patent did not in fact teach a consensus human variable domain as the term is used in the present application.

Applicants contend that the phrase "consensus framework from many human antibodies" in line 7 of column 13 in the '762 patent which is cited by the Office, was not intended to refer to a "consensus human variable domain" as in the present application (*i.e.* a sequence representing the most frequently occurring amino acid residues at each location in all immunoglobulins of any particular subclass; see page 14, lines 29-31 of the instant application). Applicants submit that the '762 patent was using the phrase "consensus framework from many human antibodies" synonymously with a framework "from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized". If one reads lines 4-26 in column 13 of the '762 patent and, indeed, the entire patent, it becomes clear that the method for humanizing advocated therein involved selecting an immunoglobulin framework sequence from a single human immunoglobulin which was unusually homologous to the donor immunoglobulin to be humanized and this is what was actually done in the working examples. It is apparent then that the phrase "consensus framework from many human antibodies" was used in the '762 patent as another way of saying "a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized", *i.e.*, a framework from a particular human immunoglobulin which "agrees" with the donor immunoglobulin when the sequences are aligned.

Thus, Applicants submit that the '762 patent did not teach or enable a consensus human variable domain as described in the present application, much less a "consensus human variable domain of a human heavy chain immunoglobulin subgroup." Accordingly,

reconsideration and withdrawal of the rejection is respectfully requested.

As to rejected claim 38, this relates to the method of "veneering" or "resurfacing" an antibody. As discussed in the above-mentioned interview, this approach was not taught in the '762 patent.

Applicants respectfully request reconsideration and withdrawal of the Section 102(e) rejection in view of the above.

Section 103

Claims 26-36 and 40-41 are rejected under 35 USC §103 as being unpatentable over the '762 patent in view of Kabat *et al*.

The Examiner asserts that the claimed invention differs from the prior art teachings only by recitation of Ig gamma isotype sequences used to make a consensus heavy chain framework region. The Examiner cites Kabat as teaching the sequences of all known Ig gamma subtypes and contends that it would have been *prima facie* obvious at the time the invention was made to use the teachings of the '762 patent and align all of the known Ig gamma heavy chains for the creation of a consensus sequence with the expectation that such consensus sequence immunoglobulin would have a smaller chance of changing an amino acid near the CDRs that distorts their conformation as allegedly taught in column 13 of the '762 patent.

Applicants submit that the instant invention is patentable over the cited art.

With respect to the Examiner's combining of the '762 patent and Kabat, Applicants submit that the rejection is made impermissibly using hindsight reconstruction of the present invention. "One cannot use hindsight reconstruction to pick and choose among

isolated disclosures in the prior art to depreciate the claimed invention." *In re Fine* 837 F2d 1071, 1075 (Fed. Cir. 1988).

In particular, as noted above, the term "consensus framework from many human antibodies" in the '762 patent was not intended to refer to a sequence representing the most frequently occurring amino acid residues at each location in all immunoglobulins of any particular subclass as in the present application. Thus, Applicants submit that the '762 patent would not have provided any motivation to make a consensus human variable domain as in the present application.

With respect to the Examiner's assertion that "the claimed invention differs from the prior art teachings only by recitation of Ig gamma isotype sequences used to make a consensus heavy chain framework region", Applicants believe that the Examiner has misunderstood the selection invention involving a "V_H subgroup III" consensus sequence. As opposed to a collection of antibodies with the same "isotype" due to the amino acid sequence of their heavy chain constant region (page 11 of the application), V_H subgroup III represents a subclass of antibodies grouped together because of their heavy chain variable domain sequences. For this reason alone, Applicants submit that the Examiner has failed to establish a *prima facie* case of obviousness.

Moreover, Applicants submit that there was nothing in the cited art to suggest combining Kabat with the '762 patent. In particular, the term "consensus" is not used in Kabat. Kabat refers to "occurrences of most common amino acid" for various heavy or light chain immunoglobulin subgroups. Without knowing about the invention of the present application, Applicants contend that those skilled in the art would not have been motivated to combine the mention of "consensus framework from many human antibodies" in the '762 patent with Kabat's disclosure of "occurrences of most common

amino acid", especially since, as elaborated above, the '762 patent did not intend the term "consensus framework" to refer to "occurrences of most common amino acid".

This further illustrates that the Examiner is using impermissible hindsight to combine the references.

Moreover, Applicants are able to show that the '762 patent would have taught away from the instantly claimed invention. In particular, the '762 patent states that one must select a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin in order to reduce the chance of distorting the conformation of the CDR's (see column 13 of the '762 patent). This has been termed the "best-fit" method of humanization.

On the contrary, the instant invention does not rely on selection of an unusually homologous framework from a single human antibody; a consensus human variable domain comprising the most frequently occurring amino acid residues at each location in human immunoglobulins is used as the framework region.

Whereas the '762 patent requires at least 65% homology between the human "acceptor" framework region (FR) sequence and murine "donor" FR sequence (see column 13, lines 33-36) to avoid distorting the conformation of the CDRs, Applicants have generated humanized antibodies using the V_H subgroup III consensus sequence having low FR homology to murine donor antibody FR sequences.

For example, in contrast to the teachings of the '762 patent, Applicants have shown that FR homologies as low as 53% for an anti-CD18 antibody (Example 4 on page 89 of the present application); 57% for an anti-IgE antibody [Presta *et al. J. Immunol.*

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151(5):2623-2632 (1993) (of record)]; 57% for an anti-CD11a antibody [Werther *et al. J. Immunol.* 157:4986-4995 (1996) (of record)]; 61% for an anti-VEGF antibody [Presta *et al. Cancer Research* 57(20):4593-4599(1997)(copy attached)] and 63% for an anti-HER2 antibody1 (Example 1 herein) have resulted in humanized antibodies with strong binding affinities.

Applicants submit that the '762 patent would have lead those skilled in the art away from the instantly claimed invention because they would have feared that this would result in "distortions in the CDR's" of the humanized antibody so produced.

In further support of the patentability of the instant claims, Applicants will now show that the claimed invention can produce humanized antibodies with at least three unexpected and useful properties. Unexpected results provide objective evidence of non-obviousness. *Specialty Composites v. Cabot Corp.*, 845 F. 2d 981, 6 USPQ 2d 1601 (Fed. Cir. 1988).

The unexpected properties to be demonstrated include: lack of significant immunogenicity of the claimed humanized antibodies upon repeated administration to a human patient, *e.g.*, to treat a chronic disease in the patient; binding affinities superior to those of the non-human parent antibody; and the ability to use the same consensus human variable domain to make many strong affinity antibodies, thus avoiding tailoring each human FR to each non-human antibody to be humanized.

In order to demonstrate that lack of significant immunogenicity upon repeated administration of the humanized antibody to a human

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1 In the case of the anti-HER2 antibody, surprisingly, the humanized antibody had improved binding affinity relative to the murine parent antibody. This unexpected result will be discussed in more detail below.

patient could not have been predicted for the instantly claimed humanized antibodies, Applicants refer to Isaacs *et al. The Lancet* 340:748-752 (1992) (of record). Isaacs *et al.* demonstrate that three out of four patients treated with humanized CAMPATH-1H antibody (*i.e.* the antibody humanized in Riechmann) developed antiglobulins that were able to inhibit the binding of CAMPATH-1H to its antigen (see first paragraph of the discussion on page 751 of this reference).

On the contrary, the instant application describes humanized antibodies which lack significant immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient. Therefore, the instantly claimed antibodies are useful, among other things, for treating chronic disorders such as cancer.

As suggested by the Examiner in the interview, Applicants attach a Declaration under 37 CFR §1.132 by Dr. Steven Shak. In his declaration, Dr. Shak discusses human clinical data which demonstrates the lack of significant immunogenicity of humanized antibodies of the present application. Dr. Shak is a very experienced clinician with over 20 years experience as is evident from his curriculum vitae attached as Exhibit A to his declaration.

Dr. Shak explains in paragraph 2 of his declaration that the instant application describes humanized antibodies which were anticipated to lack significant immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient.

Dr. Shak further states that the humanized anti-HER2 antibody, huMAb4D5-8 (HERCEPTIN®), disclosed in Example 1 of the above-

identified patent application has been repeatedly administered to patients in breast cancer clinical trials (paragraph 3 of the declaration). Using an ELISA to detect antibodies to HERCEPTIN® antibody in the serum of treated patients, Dr. Shak reports in paragraph 4 that only one patient out of the 885 patients evaluated as of December 31, 1997 had detectable human antihuman antibodies (HAHA).

Dr. Shak further reviews in paragraphs 5-7 of his declaration human clinical data relating to a humanized variant of a murine anti-IgE antibody which was humanized according to the teachings of the present application. Dr. Shak explains that human patients suffering from allergic rhinitis and asthma (both chronic diseases) have received repeated administrations of the humanized anti-IgE antibody (rhuMAb-E25), but no patients were found to have HAHA to rhuMAb-E25. This is particularly impressive given that the patients who were treated with rhuMAb-E25 were hyper-reactive to foreign antigens.

Dr. Shak states in the final two paragraphs of his declaration that no significant immunogenic response has been observed in patients treated with two further antibodies which were humanized according to the teachings of the present application; *i.e.*, anti-VEGF and anti-CD11a (paragraphs 8 and 9 of the declaration). The patients received multiple doses of these two antibodies.

Accordingly, Applicants submit that it is apparent that the instant specification describes humanized antibodies which lack significant immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient.

In accordance with a recommendation of the Examiner in the interview, for clarity reasons, independent claim 106 herein

includes functional language concerning the unexpected lack of significant immunogenicity of the antibody claimed therein.

In addition to the above-discussed unexpected result pertaining to lack of immunogenicity of the humanized antibodies of the present invention, binding affinity is essentially retained and in some instances is surprisingly improved in the humanized antibody compared to the non-human parent antibody. As shown, for example, in the second to last column of Table 3 on page 72, anti-HER2 humanized variants huMAb4D5-6 and huMAb4D5-8 had binding affinities which were superior to the non-human parent antibody. This could not have been predicted from the prior art, especially from the '762 patent, which advocated the best-fit method (see above) to generate a "high affinity" humanized antibody. The above-mentioned anti-HER2 variants on the other hand were not generated using the "best-fit" method said to be essential in the '762 patent.

As suggested by the Examiner in the interview, claim 113 herein refers to this unexpected property of the humanized variant in that claim (*i.e.* a variant which binds an antigen with better affinity than the non-human parent antibody).

The '762 patent fails to teach humanized antibodies which bind antigen with better affinity than the parent antibody. The reported affinity comparisons in the '762 patent are summarized here for the Examiner's convenience:

- The humanized anti-Tac antibody in Example 1 of the '762 patent allegedly had "approximately the same" binding affinity as the murine parent anti-Tac antibody (lines 25-31 in column 41). The corresponding scientific publication, Queen *et al. PNAS (USA)* 86:10029-10033 (1989) (of record) states that the humanized

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anti-Tac antibody actually had an affinity about 1/3 that of murine anti-Tac (see the abstract).

- The humanized mik-β1 humanized antibody of Example 5 had a binding affinity 2-fold worse than the mouse mik-β1 antibody (lines 50-52 in column 52 and Figure 28).

- The humanized Fd79 antibody of the '762 patent apparently displayed a 2-fold decrease in affinity and the affinity of the humanized Fd138-80 antibody was apparently "comparable" to that of the murine antibody (lines 42-46 in column 56).

- The humanized M195 antibody is stated to have an "affinity the same as the mouse M195 antibody to within experimental error" (lines 31-32 in column 60).

- In the line bridging columns 63-64, the humanized CMV5 antibody is stated to have "approximately the same binding affinity as mouse CV5".

- Finally, lines 9-11 in column 67 state that "Mouse AF2 and humanized AF2 will compete similarly, showing that their binding affinities for γ-IFN are approximately the same".

Hence, the '762 patent, in addition to its deficiencies with respect to the use of a consensus human variable domain as in the present application, fails to report any humanized antibody with better binding affinity than the non-human parent antibody.

With respect to another unexpected feature of the present invention, Applicants have shown that a consensus human variable domain of a human heavy chain immunoglobulin subgroup can be used to generate many different strong affinity humanized antibodies, including the following:

(a) anti-HER2 (4D5) [see Example 1 of the application];

(b) anti-CD3 [see Example 3 of the application];

(c) anti-CD18 [see Example 4 of the application];

(d) anti-IgE [see Presta *et al. J. Immunol.* 151(5):2623-2632 (1993) (of record)];

(e) anti-CD11a [see Werther *et al. J. Immunol.* 157:4986-4995 (1996) (of record)]; and

(f) anti-VEGF [see Presta *et al. Cancer Research* 57(20): 4593-4599 (1997) (copy attached]

This could not have been predicted based on the teachings of the '762 patent, since this reference taught that an individual human framework region needed to be tailored to each non-human antibody to be humanized (see comments above).

In summary then, Applicants submit that the cited art is deficient in teaching the instantly claimed humanized antibodies and the unexpected results of the present invention.

Turning now to claim 111 herein, this claim recites the selection invention concerning a "V_H subgroup III" consensus sequence. Applicants submit that this claim is independently patentable.

In particular, there is no suggestion in the cited art to use the particular V_H subgroup III consensus sequence.

In fact, the '762 patent taught away from this consensus sequence by advocating the "best-fit" method of humanization using the most homologous human framework for humanization. As noted above, the V_H subgroup III consensus sequence lacks significant homology to the various non-human antibodies humanized according to the teachings of the present invention. Even if (which is strongly

denied), the '762 patent had intended the phrase "consensus framework from many human antibodies" in column 13 thereof to mean a consensus human variable domain as contemplated in the present application, there is nothing in the '762 patent to indicate that a useful consensus sequence is that of a human heavy chain immunoglobulin subgroup in Kabat, let alone V_H subgroup III. For example, even though the V_H subgroup I FR in Kabat was more homologous (67% homology) to the murine anti-HER2 antibody 4D5 in Example 1 than the V_H subgroup III FR (63% homology), the inventors did not use the more homologous consensus sequence. Notwithstanding this, humanized anti-HER2 antibodies produced using this low homology human FR bound target antigen with <u>better</u> affinity than the non-human parent antibody (see comments above).

Moreover, Applicants have subsequently found that V_H subgroup III consensus sequence surprisingly has the same amino acid sequence as the human germline sequence YAC-5 in Fig. 2 of Cook *et al.*, *Nature Genetics* 7:162-168 (1994) (of record). This subsequent finding supports Applicants' observations that antibodies humanized using this FR sequence are non-immunogenic in humans.

In summation then, Applicants submit that there is nothing in the cited references to teach selection of a V_H subgroup III consensus sequence as in claim 111 for forming the V_H FR template of the humanized antibody, much less the advantages associated with such a consensus sequence. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Applicants believe that this case is now in condition for allowance and look forward to receiving early notification of same. If there are outstanding issues however, Applicants invite the Examiner to call the undersigned at the number noted below.

Respectfully submitted,

GENENTECH, INC.

Date: August 24, 1998 By: _____

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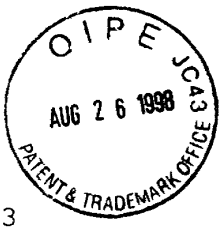
PATENT
Docket P709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
)
Carter et al.)
)
Serial. No. 08/146,206)
)
Filed: 17 November 1993)
)
For: Method for Making Humanized Antibodies



Group Art Unit: 1644

Examiner: P. Nolan

DECLARATION UNDER 37 CFR §1.132

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, STEVEN SHAK, do hereby declare and say as follows:

1. I obtained my M.D. degree in 1977 from New York University (NYU) School of Medicine. Following this, I was a Teaching Assistant and then an Assistant Professor of Medicine and Pharmacology at NYU School of Medicine. Since 1986, I have been employed as a Scientist at Genentech, Inc. Presently, I am the Clinical Team Leader for the therapeutic antibody, anti-HER2. A complete listing of my professional experience, project management experience, education, postdoctoral training, certification and licensure, honors and awards, and publications is found in my curriculum vitae attached as Exhibit A.

2. In my capacity as anti-HER2 Clinical Team Leader, I am familiar with human clinical data relating to the humanized anti-HER2 antibody, huMAb4D5-8 (HERCEPTIN®), disclosed in Example 1 of the above-identified patent application. As explained on page 70,

lines 7-9 of the above application, a humanized variant of the murine anti-HER2 antibody was made which was intended to lack significant immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient.

3. The HERCEPTIN® antibody has been administered to patients in breast cancer clinical trials using a dosing protocol which involves an initial loading dose of 4mg/kg of intravenous (IV) HERCEPTIN® antibody then weekly doses of 2mg/kg (IV) each. Patients have been treated with HERCEPTIN® antibody as a single agent or HERCEPTIN® antibody concomitantly with either (a) cyclophosphamide and doxorubicin or epirubicin (AC) or (b) paclitaxel (TAXOL®).

4. The presence of antibodies to HERCEPTIN® antibody in the serum of treated patients has been determined by enzyme-linked immunosorbent assay (ELISA). As of December 31, 1997, there is only one case of human antihuman antibodies (HAHA) in 885 patients evaluated. This one patient received nine weekly infusions of HERCEPTIN® antibody and discontinued the study on day 65 due to disease progression. At the termination evaluation, antibody measurements were suggestive of antibody formation against the $F(ab')_2$ portion of the HERCEPTIN® antibody. Antibody formation in this one case was not associated with severe allergic symptoms.

5. I have also reviewed human clinical data in relation to a humanized variant of the murine antibody MaE11 which binds IgE. MaE11 was humanized using a consensus human variable domain of a human heavy chain immunoglobulin subgroup [see Figure 1 of Presta *et al. J. Immunol.* 151(5):2623-2632 (1993), Exhibit B attached].

6. Recombinant humanized MaE11 (rhuMAb-E25) has been administered intravenously (IV) or subcutaneously (SQ) to human

patients suffering from allergic rhinitis and asthma. One hundred eighty one subjects with a documented history of seasonal allergic rhinitis or rhinoconjunctivitis received an initial IV loading dose followed by SQ or IV administrations of rhuMAb-E25 on days 7, 14, 28, 42, 56, 70 and 84 [Abstract of Casale *et al. J. Allergy Clin. Immunol.* 100(1):110-121 (1997); Exhibit C attached]. Nineteen allergic asthmatic subjects received rhuMAB-E25 IV the day after the baseline airway allergen challenge and at weekly intervals for eight weeks [Abstract and Figure 1 of Fahy *et al. Am J. Respir. Crit. Care Med.* 155:1828-1834 (1997); Exhibit D]. Potential HAHA in the serum of treated patients were assayed as described in Casale *et al.* and Fahy *et al.*

7. As reported on page 116 of Casale *et al.* and page 1830 of Fahy *et al.*, no patients were found to have HAHA to rhuMAb-E25.

8. I am also aware that we have not observed a significant immunogenic response in patients receiving multiple doses of a humanized anti-VEGF antibody for inhibiting VEGF-induced angiogenesis. The humanized antibody is question is a variant of murine anti-VEGF antibody A.4.6.1, and was humanized using a consensus human variable domain of a human heavy chain immunoglobulin subgroup [Figure 1 on page 4596 of Presta *et al. Cancer Research* 57(20):4593-4599 (1997); Exhibit E attached].

9. Finally, I have been told that no significant immunogenicity has been associated with repeated administration of a humanized anti-CD11a antibody to psoriasis patients. The humanized anti-CD11a antibody with which the psoriasis patients have been treated was prepared from the murine MHM24 antibody using a consensus human variable domain of a human heavy chain immunoglobulin subgroup [Figure 1 of Werther *et al. J. Immunol.* 157(11):4986-4995(1996), Exhibit F attached].

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 8/24/98

STEVEN SHAK

CURRICULUM VITAE

Steven Shak, M.D.

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Professional Experience:

1998-	Staff Clinical Scientist, Genentech, Inc.
1996-98	Senior Clinical Scientist, Genentech, Inc.
1989-96	Director, Departments of Immunobiology, Pulmonary Research, and Pathology, Genentech, Inc.
1986-89	Scientist, Genentech, Inc.
1984-86	Assistant Professor of Medicine and Pharmacology New York University School of Medicine
1978-80	Teaching Assistant, Department of Medicine New York University School of Medicine

Project Management:

1996-	Anti-HER2 Clinical Team Leader
1996-97	Anti-VEGF Clinical Team Leader
1996-	Chair, Clinical Assessment Committee
1993-96	Chair, Genentech-GenVec Research Committee
1993-	Board of Directors, Genentech Endowment for Cystic Fibrosis
1991-96	Research Representative on Clinical Research Advisory Committee
1995-96	DNase SLE Biology Team Leader
1992-94	DNase Pulmozyme Chronic Bronchitis Team Leader

EXHIBIT A

1988-91	DNase Pulmozyme Project Team Leader

Education:

1973-77	M.D., New York University School of Medicine
1969-73	B.A., Amherst College

Postdoctoral Training:

Research:	
1981-84	University of California, San Francisco Cardiovascular Research Institute Rosalyn Russell Arthritis Research Laboratory Chief: Ira M. Goldstein, M.D.
Fellowship:	
1980-84	University of California, San Francisco Cardiovascular Research Institute Subspeciality: Pulmonary Medicine Chairmen: John F. Murray, M.D. and Jay A. Nadel, M.D.
Residency:	
1977-80	Bellevue Hospital Specialty: Internal Medicine Chairman: Saul J. Farber, M.D.

Certification and Licensure:

1982	Diplomate, Pulmonary Disease
1980	Diplomate, American Board of Internal Medicine
1980	Licensed, California (current)
1978	Licensed, New York State

Honors and Awards:

1995	Prix Gallien, Portugal for "Pulmozyme Discovery and Development"
1995	"Parenting Achievement Award," Parenting Magazine
1993	Distinguished Corporate Scientist Award, Cystic Fibrosis

Foundation

1992	CF Achievement Award, Cystic Fibrosis Research, Inc.
1985	J. Burns Amberson Award, NY Lung Association
1980	Medical School Pulmonary Faculty Training Award
	National Institutes of Health
1977	Alpha Omega Alpha
1974	Valentine Mott Award in Anatomy and Cell Biology
1973	Summa Cum Laude
1973	Phi Beta Kappa
1973	Sigma Xi
1973	Howard Waters Doughty Prize in Chemistry

Personal:

Born: July 21, 1950, Elizabeth, NJ
Married, two children
Social Security No.: 145-42-8006

Publications:

I. Book Chapters.

1. SHAK S, Goldstein IM: The major pathway for leukotriene B_4 catabolism in human polymorphonuclear leukocytes involves ω-oxidation by a cytochrome P-450 enzyme. In PROSTAGLANDINS, LEUKOTRIENES, AND LIPOXINS. (JM Bailey, ed.) Plenum Publishing Corporation, New York, 1985.

2. SHAK S: Leukotriene B_4 catabolism: Quantitation of leukotriene B_4 and its ω-oxidation prducts by reversed phase high-performance liquid chromatography. METHODS IN ENZYMOLOGY. Vol. 141. Cellular Regulators (AR Means and PM Conn, eds.) Academic Press, Florida, pp. 355-371, 1987.

3. SHAK S: Molecular mechanisms for the catabolism of leukotriene B_4. In ADVANCES IN INFLAMMATION RESEARCH. Vol. 12. (A Lewis, ed.) Raven Press, Ltd., New York, pp. 111-124, 1988.

4. Goldstein IM, SHAK S: Humoral and cellular mediators of host defenses. In TEXTBOOK OF RESPIRATORY MEDICINE. (JF Murray and JA Nadel, eds.) W.B. Saunders Company, Philadelphia, pp. 358-373, 1988.

5. Goldstein IM, SHAK S: Host defenses in the lung: Neutrophils, complement, and other humoral mediators. In TEXTBOOK OF RESPIRATORY MEDICINE. (JF Murray and JA Nadel, eds.) W.B. Saunders Company, Philadelphia, pp. 402-418, 1994.

6. S SHAK: Mucins and lung secretions. In THE LUNG--SCIENTIFIC FOUNDATIONS. (RG Crystal, JB West, ER Weibel, and PJ Barnes, eds.) Lippincott-Raven Publishers, Philadelphia, pp. 479-486.

II. Articles

1. SHAK, S, Perez HD, Goldstein IM: A novel dioxygenation product of arachidonic acid posseses potent chemotactic activity for human polymorphonuclear leukocytes. THE JOURNAL OF BIOLOGICAL CHEMISTRY, 258:14948-14953, 1983.

2. Perez HD, Bissell DM, Roll FJ, SHAK S, Goldstein IM: A possible explanation for leukocytic infiltration of the liver in acute alcoholic hepatitis: Ethanol-induced generation by hepatocytes of a lipid chemotactic factor. TRANSACTIONS OF THE ASSOCIATION OF AMERICAN PHYSICIANS. 96:56-64, 1983.

3. Charo, IF, SHAK S, Darasek MA, Davison PM, Goldstein IM: Prostaglandin I_2 is not a major metabolite of arachidonic acid in cultured endothelial cells from human foreskin microvessels. THE JOURNAL OF CLINICAL INVESTIGATION. 74:914-919, 1984.

4. Perez HD, Roll JF, Bissell DM, SHAK S, Goldstein IM: Ethanol induces isolated rat hepatocytes to generate chemotactic activity for polymorphonuclear leukocytes. THE JOURNAL OF CLINICAL INVESTIGATION. 74:1350-1357, 1984.

5. SHAK S, Goldstein IM: ω-Oxidation is the major pathway for the catabolism of leukotriene B_4 in human polymorphonuclear leukocytes. THE JOURNAL OF BIOLOGICAL CHEMISTRY. 259:10181-10187, 1984.

6. SHAK S, Goldstein IM: Carbon monoxide inhibits ω-oxidation of leukotriene B_4 by human polymorphonuclear leukocytes: Evidence that catabolism of leukotriene B_4 is mediated by a cytochorme P-450 enzyme. BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. 123:475-481, 1984.

7. SHAK S, Reich N, Goldstein IM, Ortiz de Montellano PM: Leukotriene B_4 ω-hydroxylase in human polymorphonuclear leukocytes: Suicidal inactivation by acetylenic fatty acids. THE JOURNAL OF BIOLOGICAL CHEMISTRY. 260:13023-13028, 1985.

8. SHAK S, Goldstein IM: Leukotriene B_4 ω-hydroxylase in human polymorphonuclear leukocytes: Partial purification and identification as a cytochrome P-450. THE JOURNAL OF CLINICAL INVESTIGATION. 76:1218-1228, 1985.

9. SHAK S, Goldstein IM: The leukotriene B_4 ω-hydroxylase in human polymorphonuclear leukocytes is a membrane-associated, NADPH-dependent cytochrome P-450 enzyme. TRANSACTIONS OF THE ASSOCIATION OF AMERICAN PHYSICIANS. 48:352-360, 1985.

10. Kruskal BA, SHAK S, Maxfield FR: Spreading of human neutrophils is immediately preceded by a large increase in cytoplasmic free calcium concentration. PROCEEDINGS OF THE NATIONAL ACADEMY OF THE SCIENCES USA. 83:2919-2923, 1986.

11. Davitz MA, Hereld D, SHAK S, Krakow JL, Englund PT, Nussenzweig V: A glycan-phosphatidylinositol-specific phospholipase D in human serum. SCIENCE. 238:81-4, 1987.

12. SHAK S, Davitz MA, Wolinsky ML, Nussenzweig V, Turner MJ, Gurnett A: Partial characterization of the cross reacting determinant, a carbohydrate epitope shared by decay accelerating factor (DAF) and the variant surface glycoprotein (VSG) of the african Trypanosoma brucei. THE JOURNAL OF IMMUNOLOGY. 140:2046-2050, 1988.

13. SHAK S, Capon DJ, Hellmiss R, Marsters SA, Baker CL: Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. PROCEEDINGS OF THE NATIONAL ACADAMY OF SCIENCES, USA. 87:9188-9192, 1990.

14. Aitken ML, Burke W, McDonald G, SHAK S, Montgomery AB, Smith A: Recombinant human DNase inhalation in normal and patients with cystic fibrosis: A phase I study. THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION. 267:1947-1951, 1992.

15. Hubbard RC, McElvaney NG, Birrer P, SHAK S, Robinson WW, Jolley C, Wu M, Chernick MS, Crystal RG: A preliminary study of aerosolized recombinant human deoxyribonuclease I in the treatment of cystic fibrosis. THE NEW ENGLAND JOURNAL OF MEDICINE. 326:812-815, 1992.

16. Ramsey BW, Astley SJ, Aitken ML, Burke W, Colin AA, Dorkin HL, Eisenberg JD, Gibson RL, Harwood IR, Schidlow DV, WilmottRW, Wohl ME, Myerson LJ, SHAK S, Fuchs H, Smith AL: Efficacy and safety of short-term administration of aerosolized recombinant human deoxyribonuclease in patients with cystic fibrosis. AMERICAN REVIEW OF RESPIRATORY DISEASE. 148:145-151, 1993.

17. Ranasinha C, Assoufi B, SHAK S, Christiansen D, Fuchs H, Empey D, Geddes D, Hodson M: Efficacy and safety of short-term administration of aerosolised recombinant human DNase I in adults with stable stage cystic fibrosis. THE LANCET. 342:199-202, 1993.

18. Chamow SM, Kogan TP, Venuti M, Gadek T, Harris RJ, Peers DH, Mordenti J, SHAK S, Ashkenazi A: Modification of CD4 immunoadhesin with monomethoxypoly(ethylene glycol) aldehyde via reductive alkylation. BIOCONJUGATE CHEMISTRY. 5:133-140, 1994.

19. Sinicropi D, Baker DL, Prince WS, Shiffer K, SHAK S: Colorimetric determination of DNase I activity with a DNA-methyl green substrate. ANALYTICAL BIOCHEMISTRY. 222:351-358, 1994.

20. SHAK S: Aerosolized recombinant human DNase I for the treatment of cystic fibrosis. CHEST 107:65S-70S, 1995.

21. Zahm JM, Girod de Bentzmann S, Deneuville E, Perrot-Minnot C, Dabadie A, Pennaforte F, Roussey M, SHAK S, Puchelle E: Dose-dependent in vitro effect of recombinant human DNase on rheological and transport properties of cystic fibrosis respiratory mucus. EUROPEAN RESPIRATORY JOURNAL. 8:381-6, 1995.

22. Puchelle E, Zahm JM, de Bentzmann S, Grosskopf C, SHAK S, Mougel D, Polu JM: Effects of rhDNase on purulent airway secretions in chronic bronchitis. EUROPEAN RESPIRATORY JOURNAL. 9:765-9, 1996.

23. Macanovic M, Sinicropi D, SHAK S, Baughman S, Thiru S, Lachmann PJ: The treatment of systemic lupus erythematosus (SLE) in NZB/W F1 hybrid mice; studies with recombinant murine DNase and with dexamethasone. CLINICAL AND EXPERIMENTAL IMMUNOLOGY. 106:243-252, 1996.

24. Ulmer JS, Herzka A, Toy KJ, Baker DL, Dodge AH, Sinicropi D, SHAK S, Lazarus RA: Engineering Actin Resistant Human DNase I for Treatment of Cystic Fibrosis. PROCEEDINGS NATIONAL ACADEMY OF SCIENCE, USA. 93:8225-8229, 1996.

#40 09/02/98
Godsm

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1644 Examiner: P. Nolan CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on August 24, 1998 Wendy M. Lee

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants submit herewith patents, publications or other information (attached hereto and listed on the attached Form PTO-1449) of which they are aware, which they believe may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 CFR §1.56.

This Information Disclosure Statement:

(a) [] accompanies the new patent application submitted herewith. 37 CFR §1.97(a).

(b) [] is filed within three months after the filing date of the application or within three months after the date of entry of the national stage of a PCT application as set forth in 37 CFR§1.491.

(c) [] as far as is known to the undersigned, is filed before the mailing date of a first Office action on the merits.

(d) [] is filed after the first Office Action and more than three months after the application's filing date or PCT national stage date of entry filing but, as far as is known to the undersigned, prior to the mailing date of either a final rejection or a notice of allowance, whichever occurs first, and is accompanied by either the fee ($240) set forth in 37 CFR §1.17(p) **or** a statement as specified in 37 CFR §1.97(e), as checked below. Should any fee be due, the U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $240.00 to cover

the cost of this Information Disclosure Statement. Any deficiency or overpayment should be charged or credited to this deposit account. **A duplicate of this sheet is enclosed.**

(e) [] is filed after the mailing date of either a final rejection or a notice of allowance, whichever occurred first, and is accompanied by the fee ($130) set forth in 37 CFR §1.17(i) **and** a statement as specified in 37 CFR §1.97(e), as checked below. **This document is to be considered as a petition requesting consideration of the information disclosure statement.** The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $130.00 to cover the cost of this Information Disclosure Statement. Any deficiency or overpayment should be charged or credited to this deposit account. **A duplicate of this sheet is enclosed.**

(f) [x] is filed after the mailing date of a final rejection, but a request to withdraw the finality thereof under 37 CFR § 1.129(a) is submitted herewith. The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 to cover the cost of this Information Disclosure Statement in the event that any fees are due. **A duplicate of this sheet is enclosed.**

[If either of boxes (d) or (e) is checked above, the following statement under 37 CFR §1.97(e) may need to be completed.] The undersigned states that:

[] Each item of information contained in the information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement.

[] No item of information contained in this information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application and, to the knowledge of the undersigned after making reasonable inquiry, was known to any individual designated in 37 CFR §1.56(c) more than three months prior to the filing of this information disclosure statement.

A list of the patent(s) or publication(s) is set forth on the attached Form PTO-1449 (Modified).

A copy of the items on PTO-1449 is supplied herewith:

[] each [] none [x] only those listed below:

5,677,171

5,772,997

Brown, Jr. et al.

Mathieson et al.

Presta et al.

Casale et al.

Fahy et al.

A concise explanation of relevance of the items listed on PTO-1449 is:

[x] not given

[] given for each listed item

[] given for only non-English language listed item(s) [Required]

[] in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references.

The Examiner is reminded that a "concise explanation of the relevance" of the submitted prior art "may be nothing more than identification of the particular figure or paragraph of the patent or publication which has some relation to the claimed invention," MPEP §609.

While the information and references disclosed in this Information Disclosure Statement may be "material" pursuant to 37 CFR §1.56, it is not intended to constitute an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

In accordance with 37 CFR §1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR §1.56(a) exists. It is submitted that the Information Disclosure Statement is in compliance with 37 CFR §1.98 and MPEP §609 and the Examiner is respectfully requested to consider the listed references.

Respectfully submitted,
GENENTECH, INC.

Date: August 24, 1998

By: _____
Wendy M. Lee
Reg. No. 40,378

1 DNA Way
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UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, DC 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.

EXAMINER	
ART UNIT	PAPER NUMBER

DATE MAILED: #41

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

PTO-90C (REV. 2/95)

1-File Copy

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.

EXAMINER	
ART UNIT	PAPER NUMBER

DATE MAILED:

INTERVIEW SUMMARY

All participants (applicant, applicant's representative, PTO personnel):

(1) Minh-Tam Davis (3) Wendy Lee

(2) Lisa Teisse (4)

Date of Interview 10/16/97

Type: ☐ Telephonic ☐ Personal (copy is given to ☐ applicant ☒ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☐ No If yes, brief description:

Agreement ☐ was reached. ☐ was not reached.

Claim(s) discussed: all pending claims

Identification of prior art discussed:

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: 1/112, 2nd para. issue of cls 43-105 with respect to binding of CDR, 2/112, 2nd para issue of cls 105-112 with respect to "significant" immunogenicity

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

1. ☐ It is not necessary for applicant to provide a separate record of the substance of the interview.

Unless the paragraph above has been checked to indicate to the contrary. A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a response to the last Office action has are ready been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW.

2. ☐ Since the Examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action. Applicant is not relieved from providing a separate record of the interview unless box 1 above is also checked.

Examiner Note: You must sign this form unless it is an attachment to another form.

FORM **PTOL-413** (REV.1-96)

Minh-Tam Davis

11/6/98

Official Document

#42

GENENTECH, INC.

1 DNA Way, South San Francisco, CA 94080-4990 Tel: 650-225-1994 Fax: 650-952-9881

FAX TRANSMISSION COVER SHEET

Date: November 6, 1998

To: Lila Feisee ~~Examiner M.T. Davis~~ *Group Art Unit: 1642 of US PTO*

Fax: (703) 308 0294 ~~4426~~

Re: U.S. Ser. No 08/146,206 *filed November 17, 1993* *(Attorney Docket No.: P0709P1)*

Sender: Wendy M. Lee

CERTIFICATION OF FACSIMILE TRANSMISSION
I hereby certify that this paper is being facsimile transmitted to the Patent and Trademark Office on the date shown below.
Ann Savelli
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Comments:

42/I

11/6/98

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Paul J. Carter et al.	Group Art Unit: 1644
Serial No.: 08/146,206	Examiner: Tam Davis
Filed: November 17, 1993	
For: METHOD FOR MAKING HUMANIZED ANTIBODIES	

SUPPLEMENTAL AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Further to the amendment dated August 24, 1998, Applicants request that the above-identified application be amended as follows:

IN THE CLAIMS:

Please amend claims 43, 72, 104-106 and 112 as follows:

I'
Sub I

43. (Amended) A humanized antibody variable domain comprising a non-human Complementarity Determining Region (CDR) which binds an antigen incorporated into a human antibody variable domain, and further comprising an 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.

I2
Sub J3

72. (Amended) An antibody which binds p185^{HER2} and comprises a humanized antibody variable domain comprising a non-human Complementarity Determining Region (CDR) which binds p185^{HER2} 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.

104. (Amended) A humanized antibody variable domain comprising a non-human Complementarity Determining Region (CDR) which binds 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.

105. (Amended) An antibody which lacks [significant] 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 and comprises a non-human Complementarity Determining Region (CDR) which binds 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.

106. (Amended) An antibody which lacks [significant] 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 and comprises a consensus human

variable domain 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 comprising a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) comprises 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.

112. (Amended) The humanized antibody of claim 111 which lacks [significant] 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.

REMARKS

The undersigned confirms having met with Examiners Davis and Feisee in the interview October 16, 1998. In that interview, the Examiners suggested that independent claims 43, 72, 104 and 105 be amended for claim precision to refer to a CDR which binds an antigen. Without acquiescing in any objection or rejection and purely to facilitate allowance, claims 43, 104 and 105 have been revised herein as recommended by the Office to refer to a CDR "which binds an antigen" and claim 72 refers to a CDR "which binds $p185^{HER2}$".

Moreover, the Examiners proposed in the interview that, for clarity reasons, claims 105, 106 and 112 (refering to antibodies with diminished immunogenicity) be revised to refer to an antibody which "lacks immunogenicity compared to a non-human

parent antibody". Without acquiescing in any objection or rejection and purely to facilitate allowance, Applicants have adopted the language proposed by the Office. Hence, the instantly claimed antibodies display significantly reduced immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient (see page 70, lines 6-8 of the instant application), as opposed to the immunogenicity observed with the prior art humanized antibody in Isaacs *et al.*, *The Lancet* 340:748-752 (1992) (see first paragraph on page 19 of the amendment dated August 24, 1998).

Applicants look forward to early receipt of a notice of allowance in the above application.

Respectfully submitted,
GENENTECH, INC.

Date: November 6, 1998

By: _____
Wendy M. Lee
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#44

Official Document - ***GENENTECH, INC.***

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FAX TRANSMISSION COVER SHEET

Date: *January 15, 1999*

To: *Examiner Julie Reeves* *Group Art Unit: 1642 of US PTO*

Fax: *(703) 308-4426*

Re: *U S. Ser. No 08/146,206* *filed November 17, 1993* *(Attorney Docket No.: P0709P1)*

Sender: *Wendy M. Lee*

CERTIFICATION OF FACSIMILE TRANSMISSION
I hereby certify that this paper is being facsimile transmitted to the Patent and Trademark Office on the date shown below.
Ann Savelli
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Comments:

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: J. Reeves

AMENDMENT TRANSMITTAL

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated as shown below

	Claims Remaining After Amendment		Highest No. Previously Paid For	Present Extra	Rate	Additional Fees
Total	86	-	72	14	$18	$252.00
Independent	9	-	7	2	$78	$156.00
__ Multiple dependent claim(s), if any					$260	$0.00
					Total Fee Calculation	$408.00

_____
x
_____

No additional fee is required.
The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $408.00. A duplicate copy of this transmittal is enclosed.
Petition for Extension of Time is enclosed.

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR 1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. A duplicate copy of this sheet is enclosed.

Respectfully submitted,
GENENTECH, INC.

Date: January 15, 1999

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paper # 44
Amdt J

Patent Docket P07099

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

1/15/99
JR

In re Application of	Group Art Unit: 1642
Paul J. Carter et al.	Examiner: Julie Reeves
Serial No.: 08/146,206	
Filed: November 17, 1993	
For: METHOD FOR MAKING HUMANIZED ANTIBODIES	

SUPPLEMENTAL AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Please amend the claims as indicated below. Pending claims which are not amended herein are marked "(Reiterated)" for the Examiner's convenience.

1 ~~44~~. (TWICE AMENDED) A humanized antibody variable domain comprising [a] non-human Complementarity Determining Region (CDR) amino acid residues which bind[s] 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.

K J1

2 ~~44~~. (AMENDED) The humanized variable domain of claim ~~43~~ 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 [was] obtained.

J2

45. (Reiterated) The humanized variable domain of claim 43 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

Duplicate

/cc

J

46. (Reiterated) The humanized variable domain of claim 43 wherein the human antibody variable domain is a consensus human variable domain.

47. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 4L has been substituted.

48. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 38L has been substituted.

49. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 43L has been substituted.

50. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 44L has been substituted.

51. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 58L has been substituted.

52. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 62L has been substituted.

53. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 65L has been substituted.

54. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 66L has been substituted.

55. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 67L has been substituted.

56. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 68L has been substituted.

57. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 69L has been substituted.

58. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 73L has been substituted.

59. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 85L has been substituted.

60. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 98L has been substituted.

61. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 2H has been substituted.

62. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 4H has been substituted.

63. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 36H has been substituted.

64. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 39H has been substituted.

65. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 43H has been substituted.

66. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 45H has been substituted.

67. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 69H has been substituted.

68. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 70H has been substituted.

69. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 74H has been substituted.

70. (Reiterated) The humanized variable domain of claim 43 wherein the residue at site 92H has been substituted.

71. (Reiterated) An antibody comprising the humanized variable domain of claim 43.

J3

72. (TWICE AMENDED) An antibody which binds $p185^{HER2}$ and comprises a humanized antibody variable domain, wherein the humanized antibody variable domain comprises [comprising a] non-human Complementarity Determining Region (CDR) amino acid residues which bind[s] $p185^{HER2}$ 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, 68L, 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.

J4

73. (AMENDED) The antibody of claim 72 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 [was] obtained.

74. (Reiterated) The antibody of claim 72 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

75. (Reiterated) The antibody of claim 72 wherein the human antibody variable domain is a consensus human variable domain.

76. (Reiterated) The antibody of claim 72 wherein the residue at site 4L has been substituted.

77. (Reiterated) The antibody of claim 72 wherein the residue at site 38L has been substituted.

78. (Reiterated) The antibody of claim 72 wherein the residue at site 43L has been substituted.

79. (Reiterated) The antibody of claim 72 wherein the residue at site 44L has been substituted.

80. (Reiterated) The antibody of claim 72 wherein the residue at site 46L has been substituted.

81. (Reiterated) The antibody of claim 72 wherein the residue at site 58L has been substituted.

82. (Reiterated) The antibody of claim 72 wherein the residue at site 62L has been substituted.

83. (Reiterated) The antibody of claim 72 wherein the residue at site 65L has been substituted.

84. (Reiterated) The antibody of claim 72 wherein the residue at site 66L has been substituted.

85. (Reiterated) The antibody of claim 72 wherein the residue at site 67L has been substituted.

86. (Reiterated) The antibody of claim 72 wherein the residue at site 68L has been substituted.

87. (Reiterated) The antibody of claim 72 wherein the residue at site 69L has been substituted.

88. (Reiterated) The antibody of claim 72 wherein the residue at site 73L has been substituted.

89. (Reiterated) The antibody of claim 72 wherein the residue at site 85L has been substituted.

90. (Reiterated) The antibody of claim 72 wherein the residue at site 98L has been substituted.

91. (Reiterated) The antibody of claim 72 wherein the residue at site 2H has been substituted.

92. (Reiterated) The antibody of claim 72 wherein the residue at site 4H has been substituted.

93. (Reiterated) The antibody of claim 72 wherein the residue at site 36H has been substituted.

94. (Reiterated) The antibody of claim 72 wherein the residue at site 39H has been substituted.

95. (Reiterated) The antibody of claim 72 wherein the residue at site 43H has been substituted.

96. (Reiterated) The antibody of claim 72 wherein the residue at site 45H has been substituted.

97. (Reiterated) The antibody of claim 72 wherein the residue at site 69H has been substituted.

98. (Reiterated) The antibody of claim 72 wherein the residue at site 70H has been substituted.

99. (Reiterated) The antibody of claim 72 wherein the residue at site 74H has been substituted.

100. (Reiterated) The antibody of claim 72 wherein the residue at site 75H has been substituted.

101. (Reiterated) The antibody of claim 72 wherein the residue at site 76H has been substituted.

102. (Reiterated) The antibody of claim 72 wherein the residue at site 78H has been substituted.

103. (Reiterated) The antibody of claim 72 wherein the residue at site 92H has been substituted.

J5

104. (TWICE AMENDED) A humanized antibody variable domain comprising [a] non-human Complementarity Determining Region (CDR) amino acid residues which bind[s] 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.

J6

105. (TWICE AMENDED) [An] 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 [and], wherein the humanized antibody comprises [a] non-human Complementarity Determining Region (CDR) amino acid residues which bind[s] 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.

106. (TWICE AMENDED) [An] 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[and], wherein the humanized antibody comprises a consensus human variable domain 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 [comprising] comprises a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) [comprises] 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.

107. (AMENDED) The humanized antibody of claim 106 comprising a non-human FR residue which noncovalently binds antigen directly.

108. (AMENDED) The humanized antibody of claim 106 comprising a non-human FR residue which interacts with a CDR.

109. (AMENDED) The humanized antibody of claim 106 comprising a non-human FR residue which [comprises] introduces a glycosylation site which affects the antigen binding or affinity of the antibody.

110. (AMENDED) The humanized antibody of claim 106 comprising a non-human FR residue which participates in the V_L-V_H interface by affecting the proximity or orientation of the V_L-V_H regions with respect to one another.

111. (AMENDED) A humanized antibody comprising a consensus human variable domain of human V_H subgroup III, wherein amino acid residues forming Complementarity Determining Regions (CDRs) thereof comprise non-human antibody amino acid residues, and further comprising a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) [comprises] 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.

112. (Reiterated) The humanized antibody of claim 111 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.

113. (AMENDED) A humanized variant of a non-human parent antibody which binds an antigen with better affinity than the parent antibody and comprises a consensus human variable domain 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 [comprising] comprises a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) [comprises] 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.

J9

114. (AMENDED) The humanized variant of claim 113 which binds the antigen at least about 3-fold more tightly than the parent antibody binds antigen.

Please add the following claims to the above-identified application:

--115. (NEW) 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 an 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.

J10

116. (NEW) The humanized variable domain of claim 115 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.

117. (NEW) The humanized variable domain of claim 115 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

118. (NEW) The humanized variable domain of claim 115 wherein the human antibody variable domain is a consensus human variable domain.

119. (NEW) The humanized variable domain of claim 115 wherein the residue at site 24H has been substituted.

120. (NEW) The humanized variable domain of claim 115 wherein the residue at site 73H has been substituted.

121. (NEW) The humanized variable domain of claim 115 wherein the residue at site 76H has been substituted.

122. (NEW) The humanized variable domain of claim 115 wherein the residue at site 78H has been substituted.

123. (NEW) The humanized variable domain of claim 115 wherein the residue at site 93H has been substituted.

J10

124. (NEW) The humanized variable domain of claim 115 which further comprises an amino acid substitution at site 71H.

125. (NEW) The humanized variable domain of claim 115 which further comprises amino acid substitutions at sites 71H and 73H.

126. (NEW) The humanized variable domain of claim 115 which further comprises amino acid substitutions at sites 71H, 73H and 78H.

127. (NEW) An antibody comprising the humanized variable domain of claim 115.

128. (NEW) 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 a Framework Region (FR) 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 humanized variant binds the antigen more tightly than the parent antibody

binds ag.

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Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: J. Reeves

CERTIFICATE OF HAND DELIVERY
I hereby certify that this correspondence is being hand delivered in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on
February 1, 1999
R. H. Mitchell

COMMUNICATION

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Further to the Supplemental Amendment fax-filed on January 15, 1999, please find enclosed priority documents USSN 07/290,975 and USSN 07/310,252 for the "PDL Patents" as promised on page 11 of that amendment.

Applicants further submit herewith a Supplemental Information Disclosure Statement. In this respect, Applicants bring to the Examiner's attention a Celltech press release entitled: "Celltech Antibody Technology Platform Further Strengthened Through New Patents in US and Europe." (Exhibit A attached) This press release refers to an allowed US "Adair" patent application. Applicants believe this US Adair patent application corresponds to WO91/09967 (of record) and EP 460,167 B1 (copy attached).

Should the Examiner have questions concerning this communication, she is invited to call the undersigned.

Respectfully submitted,
GENENTECH, INC.

Date: January 29, 1999

By: _____
Wendy M. Lee
Reg. No. 40,378

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Celltech Antibody Technology Platform Further Strengthened Through New Patents in US and Europe

SLOUGH, U.K., Sept. 26 /PRNewswire/ -- Celltech announced today that the U.S. Patent Office has allowed one of its key patent applications covering engineered human antibodies. The grant of this "Adair" patent will occur in early 1997 and will expire in 2014. This broad product patent covers a key approach to the construction of new human antibodies which is essential in order to achieve full therapeutic activity. It covers all antibodies which have been constructed using this approach. A corresponding patent has already been granted in Europe, although it is anticipated that the financial benefit to Celltech from the U.S. patent will be more significant in the near term because of the numbers of antibodies in late-stage development in the U.S.

The "Adair" patent is an important new element in Celltech's technology platform, and complements previous Celltech patents in the field of antibody engineering. It covers all of Celltech's own antibodies currently in clinical development, thus substantially extending their period of patent protection. In addition the patent covers a range of antibodies under development by other companies. This would result in royalty revenues should these products reach the market.

There are already a number of process patents covering the manufacture of engineered antibodies including those granted to Celltech, Genentech, the Medical Research Council and Protein Design Laboratories. Celltech has agreements in place with Genentech and the Medical Research Council relating to the commercial exploitation of some of these patents. Celltech pursues the strategy of licensing its existing antibody patents to any interested party for products which are not directly competitive with Celltech's own products. This policy will be pursued with the new "Adair" patent and all licensees who have directly licensed pre-existing patents from Celltech (in particular the "Boss" antibody engineering patents) will be offered favorable terms for the "Adair" patent.

Commenting on the news today, Dr. Peter Fellner, CEO, said, "Celltech has built a very valuable platform technology in the field of antibody engineering and the grant of this patent will further strengthen our position. We expect a continued growth in royalty revenues from our licensed patents which will make a significant contribution to the profitability of the company. The potential in this area can be seen from the growing success of ReoPro(TM) (Lilly/Centocor). Royalties on the sales of this product are paid to both Genentech and Celltech." SOURCE Celltech Therapeutics Ltd.

/NOTE TO EDITORS:

1. The Adair product patent covers any antibody in which the antigen binding regions from a donor antibody have been transferred to the framework of a human antibody, and specifies certain requirements in specific amino acid residues within the product which are necessary to recover full antigen binding activity of the newly created antibody.

2. Antibodies are natural proteins which bind tightly and specifically to antigens. This binding property is particularly important in providing a defense mechanism against infectious organisms such as bacteria and viruses. For some time, scientists have been able to produce antibodies in the

laboratory and their availability has had a profound impact on diagnostic medicine. In contrast, they have had little impact on therapeutic medicine. The reason for this is that the first antibodies were derived from animal sources. When these animal antibodies were injected into humans they induced a significant immune response which led to either adverse reactions or a rapid loss of therapeutic efficacy. More recently techniques have been developed to produce engineered human antibodies which are virtually identical to natural human antibodies. The main advantage of these antibodies is that they do not cause a significant immune response in man and they are very well tolerated. Because of their good tolerance, their binding properties are being used in a wide variety of therapeutic applications in areas such as blockade of receptor functions in heart disease, neutralization of cytokine in rheumatoid arthritis and killing of cancer cells./

/CONTACT: Dr. David Bloxham, Chief Executive of Celltech Therapeutics Ltd., or Peter Allen, Finance Director of Celltech Group plc, 0-1753-534655; or Jon Coles of Brunswick, 0-171-404-5959; or Rich Tammero of Noonan/Russo Communications, Inc., 212-696-4455 ext. 222, e-mail: news@noonanrusso.com/ 08:52 EDT

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09/146206 attached

SERIAL NUMBER	PATENT DATE	PATENT NUMBER
07/290975		PCT/US 89/05857

SERIAL NUMBER	FILING DATE	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
07/290,975	12/28/88	435		185	

APPLICANTS

CARY L. QUEEN, PALO ALTO, CA; HAROLD E. SELICK, BELMONT, CA.

CONTINUING DATA********
VERIFIED

REC'D 28 DEC 1989
WIPO PCT

FOREIGN/PCT APPLICATIONS********
VERIFIED

PRIORITY DOCUMENT

FOREIGN FILING LICENSE GRANTED 01/24/89 ***** SMALL ENTITY *****

Foreign priority claimed ☐yes ☐no / 35 USC 119 conditions met ☐yes ☐no	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
Verified and Acknowledged Examiner			10	26	8	$ 396.00	118230

ADDRESS

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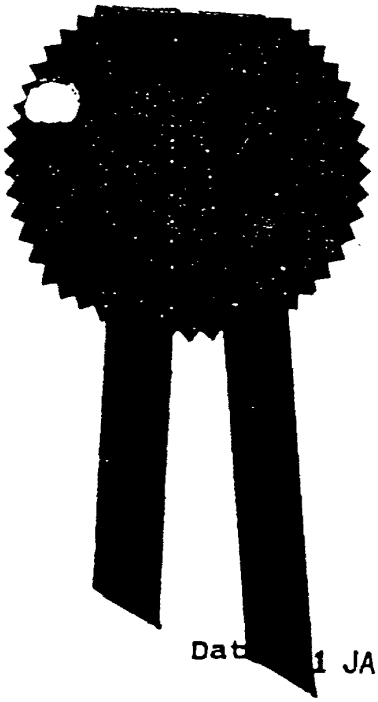
TITLE

NOVEL IL-2 RECEPTOR-SPECIFIC HUMAN IMMUNOGLOBULINS



This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application as originally filed which is identified above.

By authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

C.A. Lane

Certifying Officer

Date 1 JAN 1990

PATENT APPLICATION SERIAL NO. 07/290975

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

G 11411 01/04/89 290975 20-1430 110 201 394.00CH

PTO-1556
(5/87)

NOVEL IL-2 RECEPTOR-SPECIFIC HUMAN IMMUNOGLOBULINS

Field of the Invention

The present invention relates generally to the combination of recombinant DNA and monoclonal antibody technologies for developing novel therapeutic agents and, more particularly, to the production of non-immunogenic antibodies specific for the human interleukin-2 receptor and their uses.

Background of the Invention

In mammals, the immune response is mediated by two types of cells that interact specifically with foreign material, i.e., antigens. One of these cell types, B-cells, are responsible for the production of antibodies. The second cell class, T-cells, include a wide variety of cellular subsets controlling the in vivo function of both B-cells and a wide variety of other hematopoietic cells, including T-cells.

One way in which T-cells exert this control is through the production of a lymphokine known as interleukin-2 (IL-2), originally named T-cell growth factor. IL-2's prime function appears to be the stimulation and maintenance of T-cells. Indeed, some immunologists believe that IL-2 may be at the center of the entire immune response (see, Farrar, J., et al., Immunol. Rev. 63:129-166 (1982), which is incorporated herein by reference).

To exert its biological effects, IL-2 interacts with a specific high-affinity membrane receptor (Greene, W., et al., Progress in Hematology XIV, E. Brown, Ed., Grune and Statton, New York (1986), at pgs. 283 ff). The human IL-2 receptor is a complex multichain glycoprotein, with one chain, known as the Tac peptide, being about 55kD in size (see, Leonard, W., et al., J. Biol. Chem. 260:1872 (1985), which is incorporated herein by reference). A gene encoding this protein has been isolated, and predicts a 272 amino acid peptide, including a 21 amino acid signal peptide (see,

Leonard, W., et al., Nature 311: 626 (1984)). The 219 NH_2-terminal amino acids of the p55 Tac protein apparently comprise an extracellular domain (see, Leonard, W., et al., Science, 230:633-639 (1985), which is incorporated herein by reference).

Much of the elucidation of the human IL-2 receptor's structure and function is due to the development of specifically reactive monoclonal antibodies. In particular, one mouse monoclonal antibody, known as anti-Tac (Uchiyama, et al., J. Immunol. 126:1393 (1981)) has shown that IL-2 receptors can be detected on T-cells, but also on cells of the monocyte-macrophage family, Kupffer cells of the liver, Langerhans' cells of the skin and, of course, activated T-cells. Importantly, resting T-cells, B-cells or circulating machrophages typically do not display the IL-2 receptor (Herrmann, et al., J. Exp. Med. 162:1111 (1985)).

The anti-Tac monoclonal antibody has also been used to define lymphocyte functions that require IL-2 interaction, and has been shown to inhibit various T-cell functions, including the generation of cytotoxic and suppressor T lymphocytes in cell culture. Also, based on studies with anti-Tac and other antibodies, a variety of disorders are now associated with improper IL-2 receptor expression by T-cells, in particular adult T-cell leukemia.

More recently, the IL-2 receptor has been shown to be an ideal target for novel therapeutic approaches to T-cell mediated diseases. It has been proposed that IL-2 receptor specific antibodies, such as the anti-Tac monoclonal antibody, can be used either alone or as an immunoconjugate (e.g., with Ricin A, isotopes and the like) to effectively remove cells bearing the IL-2 receptor. These agents can, for example, theoretically eliminate IL-2 receptor-expressing leukemic cells, certain B-cells, or activated T-cells involved in a disease state, yet allow the retention of mature normal T-cells and their precursors to ensure the capability of mounting a normal T-cell immune response as needed. In general, most other T-cell specific agents can destroy essentially all peripheral T-cells, which limits the

agents' therapeutic efficacy. Overall, the use of appropriate monoclonal antibodies specific for the IL-2 receptor may have therapeutic utility in autoimmune diseases, organ transplantation and any unwanted response by activated T-cells. Indeed, clinical trials have been initiated using, e.g., anti-Tac antibodies (see, generally, Waldman, T., et al., Cancer Res. 45:625 (1985) and Waldman, T., Science 232:727-732 (1986), both of which are incorporated herein by reference).

Unfortunately, the use of the anti-Tac and other non-human monoclonal antibodies have certain drawbacks, particularly in repeated therapeutic regimens as explained below. Mouse monoclonal antibodies, for example, do not fix human complement well, and lack other important immunoglobulin functional characteristics when used in humans.

Perhaps more importantly, anti-Tac and other non-human monoclonal antibodies contain substantial stretches of amino acid sequences that will be immunogenic when injected into a human patient. Numerous studies have shown that, after injection of a foreign antibody, the immune response elicited by a patient against an antibody can be quite strong, essentially eliminating the antibody's therapeutic utility after an initial treatment. Moreover, as increasing numbers of different mouse or other antigenic (to humans) monoclonal antibodies can be expected to be developed to treat various diseases, after the first and second treatments with any different non-human antibodies, subsequent treatments even for unrelated therapies can be ineffective or even dangerous in themselves.

While the production of so-called "chimeric antibodies" (e.g., mouse variable regions joined to human constant regions) has proven somewhat successful, a significant immunogenicity problem remains. In general, the production of human immunoglobulins reactive with the human IL-2 receptor, as with many human antigens, has been extremely difficult using typical human monoclonal antibody production techniques. Similarly, utilizing recombinant DNA

technology to produce so-called "humanized" antibodies (see, e.g., EPO Publication No. 0239400), provides uncertain results, in part due to unpredictable binding affinities.

Thus, there is a need for improved forms of human-like immunoglobulins specific for the human IL-2 receptor that are substantially non-immunogenic in humans, yet easily and economically produced in a manner suitable for therapeutic formulation and other uses. The present invention fulfills these and other needs.

Summary of the Invention

The present invention provides novel compositions useful, for example, in the treatment of T-cell mediated human disorders, the compositions containing human-like immunoglobulins specifically capable of blocking the binding of human IL-2 to its receptor and/or capable of binding to the p55 Tac protein on human IL-2 receptors. The immunoglobulins can have two pairs of light chain/heavy chain complexes, typically at least one pair having chains comprising mouse complementarity determining regions functionally joined to human framework region segments. For example, mouse complementarity determining regions, with or without additional naturally-associated mouse amino acid residues, can be used to produce human-like antibodies capable of binding to the human IL-2 receptor at affinity levels stronger than about 10^8 M^{-1}.

The immunoglobulins, including binding fragments and other derivatives thereof, of the present invention may be produced readily by a variety of recombinant DNA techniques, with ultimate expression in transfected cells, preferably immortalized eukaryotic cells, such as myeloma or hybridoma cells. Polynucleotides comprising a first sequence coding for human-like immunoglobulin framework regions and a second sequence set coding for the desired immunoglobulin complementarity determining regions can be produced synthetically or by combining appropriate cDNA and genomic DNA segments.

The human-like immunoglobulins may be utilized alone in substantially pure form, or complexed with a cytotoxic agent, such as a radionuclide, a ribosomal inhibiting protein or a cytotoxic agent active at cell surfaces. All of these compounds will be particularly useful in treating T-cell mediated disorders. The human-like immunoglobulins or their complexes can be prepared in a pharmaceutically accepted dosage form, which will vary depending on the mode of administration.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Comparison of sequences of anti-Tac heavy chain (upper lines) and Eu heavy chain (lower lines). The 1-letter code for amino acids is used. The first amino acid on each line is numbered at the left. Identical amino acids in the two sequences are connected by lines. The 3 CDRs are underlined. Other amino acid positions for which the anti-Tac amino acid rather than the Eu amino acid was used in the humanized anti-Tac heavy chain are denoted by an *.

Figure 2. Comparison of sequences of anti-Tac light chain (upper lines) and Eu light chain (lower lines). The single-letter code for amino acids is used. The first amino acid on each line is numbered at the left. Identical amino acids in the two sequences are connected by lines. The 3 CDRs are underlined. Other amino acid positions for which the anti-Tac amino acid rather than the Eu amino acid was used in the humanized anti-Tac heavy chain are denoted by an *.

Figure 3. Nucleotide sequence of the gene for the humanized anti-Tac heavy chain variable region gene. The translated amino acid sequence for the part of the gene encoding protein is shown underneath the nucleotide sequence. The nucleotides TCTAGA at the beginning and end of the gene are Xba I sites. The mature heavy chain sequence begins with amino acid #20 Q.

Figure 4. Nucleotide sequence of the gene for the humanized anti-Tac light chain variable region gene. The translated amino acid sequence for the part of the gene encoding protein is shown underneath the nucleotide sequence. The nucleotides TCTAGA at the beginning and end of the gene are Xba I sites. The mature light chain sequence begins with amino acid #21 D.

Figure 5. A. Sequences of the four oligonucleotides used to synthesize the humanized anti-Tac heavy chain gene, printed 5' to 3'. B. Relative positions of the oligonucleotides. The arrows point in the 3' direction for each oligonucleotide.

Figure 6. (A) Sequences of the four oligonucleotides used to synchesize the humanized anti-Tac light chain gene, printed 5' to 3'. (B) Relative positions of the oligonucleotides. The arrows point in the 3' direction for each oligonucleotide. The position of a Hind III site in the overlap of JFD2 and JFD3 is shown.

Figure 7. Schematic diagram of the plasmid pHuGTAC1 used to express the humanized anti-Tac heavy chain. Relevant restriction sites are shown, and coding regions of the heavy chain are displayed as boxes. The direction of transcription from the immunoglobulin (Ig) promoter is shown by an arrow. E_H = heavy chain enhancer, Hyg = hygromycin resistance gene.

Figure 8. Schematic diagram of the plasmid pHuLTAC used to express the humanized anti-Tac light chain. Relevant restriction sites are shown, and coding regions of the light chain are displayed as boxes. The direction of transcription from the Ig promoter is shown by an arrow.

Figure 9. Fluorocytometry of HUT-102 and Jurkat cells stained with anti-Tac antibody or humanized anti-Tac antibody followed respectively by fluorescein-conjugated goat anti-mouse Ig antibody or goat anti-human Ig antibody, as labeled. In each panel, the dotted curve shows the results when the first antibody was omitted, and the solid curve the results when first and second (conjugated) antibodies were included as described.

Figure 10. (A) Fluorocytometry of HUT-102 cells stained with 0-40 ng of anti-Tac as indicated, then with biotinylated anti-Tac, and then with phycoerythrin-conjugated avidin. (B) Fluorocytometry of HUT-102 cells stained with the indicated antibody, then with biotinylated anti-Tac, and then with phycoerythrin-conjugated avidin.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, human-like immunoglobulins specifically reactive with the IL-2 receptor on human T-cells are provided. These immunoglobulins, which have binding affinities of at least about 10^8 M^{-1}, and preferably 10^9 M^{-1} to 10^{10} M^{-1} or stronger, are capable of, e.g., blocking the binding of IL-2 to human IL-2 receptors. The human-like immunoglobulins will have a human-like framework and can have complementarity determining regions (CDR's) from an immunoglobulin, typically a mouse immunoglobulin, specifically reactive with an epitope on p55 Tac protein. The immunoglobulins of the present invention, which can be produced economically in large quantities, find use, for example, in the treatment of T-cell mediated disorders in human patients by a variety of techniques.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25kD) and one "heavy" chain (about 50-70kD). The NH_2-terminus of each chain begins a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The COOH terminus of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified (and subclassified) as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 12 more amino acids. (See, generally, Fundamental Immunology, Paul, W., Ed., Chapter 7, pgs. 131-166, Raven Press, N.Y. (1984), which is incorporated herein by reference.)

The variable regions of each light/heavy chain pair form the antibody binding site. The chains all exhibit the same general structure of relatively conserved framework

regions joined by three hypervariable regions, also called CDR's (see, "Sequences of Proteins of Immunological Interest," Kabat, E., et al., U.S. Department of Health and Human Services, (1983); and Cholthia and Lesk, J. Mol. Biol., 196:901-917 (1987), which are incorporated herein by reference). The CDR's from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. The immunoglobulins may exist in a variety of forms besides antibodies; including, for example, Fv, Fab, and $F(ab)_2$, as well as in single chains (e.g., Huston, et al., Proc. Nat. Acad. Sci. U.S.A., 85:5879-5883 (1988) and Bird, et al., Science, 242:423-426 (1988), which are incorporated herein by reference). (See, generally, Hood, et al., "Immunology", Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, Nature, 323:15-16 (1986), which are incorporated herein by reference).

Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to human constant (C) segments, such as γ_1 and γ_3. A typical therapeutic chimeric antibody is thus a hybrid protein consisting of the V or antigen-binding domain from a mouse antibody and the C or effector domain from a human antibody (e.g., A.T.C.C. Accession No. CRL 9688 secretes an anti-Tac chimeric antibody), although other mammalian species may be used.

As used herein, the term "framework region" refers to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved (i.e., other

than the CDR's) among different immunoglobulins in a single species, as defined by Kabat, et al., op. cit. As used herein, a "human-like framework region" is a framework region that in each existing chain comprises at least about 70 or more amino acid residues, typically 75 to 85 or more residues, identical to those in human immunoglobulins.

As used herein, the term "human-like immunoglobulin" refers to an immunoglobulin comprising a human-like framework and in which any constant region present is substantially homologous to a human immunoglobulin constant region, i.e., at least about 85-90%, preferably about 95% identical. Hence, all parts of a human-like immunoglobulin, except possibly the CDR's, are substantially homologous to corresponding parts of one or more native human immunoglobulin sequences. For example, a human-like immunoglobulin would not encompass a chimeric mouse variable region/human constant region antibody.

Human-like antibodies have at least three potential advantages over mouse or and in some cases chimeric antibodies for use in human therapy:

1) because the effector portion is human, it may interact better with the other parts of the human immune system (e.g., destroy the target cells more efficiently by complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC)).

2) The human immune system should not recognize the framework or C region of the human-like antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody.

3) Injected mouse antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of normal antibodies (Shaw, D., et al., J. Immunol. 138:4534-4538 (1987)). Injected human-like

antibodies will presumably have a half-life essentially identical to naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDR's from an immunoglobulin capable of binding to a desired epitope on the human IL-2 receptor, such as the anti-Tac monoclonal antibody. The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate human-like framework regions. The preferred DNA sequences, which on expression code for the polypeptide chains comprising the anti-Tac heavy and light chain hypervariable regions (with human-like framework regions), are shown in Figures 1 and 2, respectively. Due to codon degeneracy and non-critical amino-acid substitutions, other DNA sequences can be readily substituted for those sequences, as detailed below.

The DNA segments will typically further include an expression control DNA sequence operably linked to the human-like antibody coding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the light chains, heavy chains, light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may follow.

Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells (see, Kabat op. cit. and WP87/02671). The CDR's for producing the immunoglobulins of the present invention will be similarly derived from monoclonal antibodies capable of binding to the

human IL-2 receptor and produced in any convenient mammalian source, including, mice, rats, rabbits, or other veterbrate capable of producing antibodies by well known methods. Suitable source cells for the DNA sequences and host cells for immunoglobulin expression and secretion can be obtained from a number of sources, such as the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," Fifth edition (1985) Rockville, Maryland, U.S.A., which is incorporated herein by reference).

In addition to the human-like immunoglobulins specifically described herein, other "substantially homologous" modified immunoglobulins can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. For example, the framework regions can vary from the sequences in Figures 3 and 4 at the primary structure level by several amino acid substitutions, terminal and intermediate additions and deletions, and the like. Moreover, a variety of different human framework regions may be used singly or in combination as a basis for the human-like immunoglobulins of the present invention. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (see, Gillman and Smith, Gene 8:81-97 (1979) and Roberts, S. et al, Nature 328:731-734 (1987), both of which are incorporated herein by reference).

Alternatively, polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities (e.g., complement fixation activity). Also because like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions from other genes (e.g., enzymes, see, commonly assigned U.S.S.N. 132,387, filed Dec. 15, 1987, which is incorporated herein by reference) to produce fusion proteins (e.g., immunotoxins) having novel properties.

The nucleic acid sequences of the present invention capable of ultimately expressing the desired human-like antibodies can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) and components (e.g., V, J, D, and C regions), as well as by a variety of different techniques. Joining appropriate genomic sequences is presently the most common method of production, but cDNA sequences may also be utilized (see, European Patent Publication No. 0239400 and Reichmann, L., et al., Nature 332:323-327 (1988), both of which are incorporated herein by reference).

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilus, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, etc, but preferably transformed B-cells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen, C., et al., Immunol. Rev. 89:49-68 (1986), which is incorporated herein by reference), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, and the like.

The vectors containing the DNA segments of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See, generally, Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, (1982), which is incorporated herein by reference.)

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin

forms of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982)). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, N.Y. (1979 and 1981)).

The antibodies of the present invention will typically find use individually in treating a T-cell mediated disease state. Generally, where the cell linked to a disease has been identified as IL-2 receptor bearing, then the human-like antibodies capable of blocking the binding of IL-2 to the human IL-2 receptor are suitable (see, U.S.S.N. 085,707, entitled "Treating Human Malignancies and Disorders," which is incorporated herein by reference).

For example, typical disease states suitable for treatment include graft versus host disease and transplant rejection in patients undergoing an organ transplant, such as heart, lungs, kidneys, liver, etc. Other diseases include autoimmune diseases, such as Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and myasthenia gravis.

The human-like antibodies of the present invention may also be used in combination with other antibodies, particularly human monoclonal antibodies reactive with other markers on cells responsible for the disease. For example, suitable T-cell markers can include those grouped into the so-called "Clusters of Differentiation," as named by the First International Leukocyte Differentiation Workshop,

Leukocyte Typing, Bernard, et al., Eds., Springer-Verlag, N.Y. (1984), which is incorporated herein by reference.

The antibodies can also be used as separately administered compositions given in conjunction with chemotherapeutic or immunosuppressive agents. Typically, the agents will include cyclosporin A or a purine analog (e.g., methotrexate, 6-mercaptopurine, or the like), but numerous additional agents (e.g., cyclophosphamide, prednisone, etc.) well-known to those skilled in the art may also be utilized.

A preferred pharmaceutical composition of the present invention comprises the use of the subject antibodies in immunotoxins. Immunotoxins are characterized by two components and are particularly useful for killing selected cells in vitro or in vivo. One component is a cytotoxic agent which is usually fatal to a cell when attached or absorbed. The second component, known as the "delivery vehicle," provides a means for delivering the toxic agent to a particular cell type, such as cells comprising a carcinoma. The two components are commonly chemically bonded together by any of a variety of well-known chemical procedures. For example, when the cytotoxic agent is a protein and the second component is an intact immunoglobulin, the linkage may be by way of heterobifunctional cross-linkers, e.g., SPDP, carbodiimide, glutaraldehyde, or the like. Production of various immunotoxins is well-known with the art, and can be found, for example in "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet," Thorpe et al, Monoclonal Antibodies in Clinical Medicine, Academic Press, pp. 168-190 (1982), which is incorporated herein by reference.

A variety of cytotoxic agents are suitable for use in immunotoxins. Cytotoxic agents can include radionuclides, such as Iodine-131, Yttrium-90, Rhenium-188, and Bismuth-212; a number of chemotherapeutic drugs, such as vindesine, methotrexate, adriamycin, and cisplatinm; and cytotoxic proteins such as ribosomal inhibiting proteins like pokeweed antiviral protein, Pseudomonas exotoxin A, ricin, diphtheria toxin, ricin A chain, etc., or an agent active at the cell surface, such as the phospholipase enzymes (e.g.,

phospholipase C). (See, generally, commonly assigned U.S.S.N. _____ (Townsend and Townsend Docket No. 11823-7-2) filed concurrently herewith, "Chimeric Toxins," Olsnes and Phil, Pharmac. Ther., 25:355-381 (1982), and "Monoclonal Antibodies for Cancer Detection and Therapy," eds. Baldwin and Byers, pp. 159-179, 224-266, Academic Press (1985), all of which are incorporated herein by reference.)

The delivery component of the immunotoxin will include the human-like immunoglobulins of the present invention. Intact immunoglobulins or their binding fragments, such as Fab, are preferably used. Typically, the antibodies in the immunotoxins will be of the human IgM or IgG isotype, but other mammalian constant regions may be utilized as desired.

The human-like antibodies and pharmaceutical compositions thereof of this invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The compositions for parenteral administration will commonly comprise a solution of the antibody or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intramuscular injection could be made up to contain 1 ml sterile buffered water, and 50 mg of antibody. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of antibody. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980), which is incorporated herein by reference.

The antibodies of this invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immune globulins and art-known lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss (e.g., with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

The compositions containing the present human-like antibodies or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the infection and the general state of the patient's own immune system, but generally range from about 1 to about 200 mg of antibody per dose, with dosages of from 5 to 25 mg per patient being more commonly used. It must be kept in mind that the materials of this invention may generally be employed in serious disease states, that is life-threatening or potentially life-threatening situations. In such cases,

in view of the minimization of extraneous substances and the lower probability of "foreign substance" rejections which are achieved by the present human-like antibodies of this invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these antibodies.

In prophylactic applications, compositions containing the present antibodies or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but generally range from 0.1 to 25 mg per dose, especially 0.5 to 2.5 mg per patient. A preferred prophylactic use is for the prevention of kidney transplant rejection.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antibody(ies) of this invention sufficient to effectively treat the patient.

Human-like antibodies of the present invention can further find a wide variety of utilities <u>in vitro</u>. By way of example, the antibodies can be utilized for T-cell typing, for isolating specific IL-2 receptor bearing cells or fragments of the receptor, for vaccine preparation, or the like.

For diagnostic purposes, the antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the human-like antibody, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the antibodies can be directly labeled. A wide variety of labels may be employed, such as radionuclides, fluors, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands (particularly haptens),

etc. Numerous types of immunoassays are available and are well known to those skilled in the art.

Kits can also be supplied for use with the subject antibodies in the protection against or detection of a cellular activity or for the presence of a selected antigen. Thus, the subject antibody composition of the present invention may be provided, usually in a lyophilized form in a container, either alone or in conjunction with additional antibodies specific for the desired cell type. The antibodies, which may be conjugated to a label or toxin, or unconjugated, are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of active antibody, and usually present in total amount of at least about 0.001% wt. based again on the antibody concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1 to 99% wt. of the total composition. Where a second antibody capable of binding to the chimeric antibody is employed in an assay, this will usually be present in a separate vial. The second antibody is typically conjugated to a label and formulated in an analogous manner with the antibody formulations described above.

The following examples are offered by way of illustration, not by limitation.

EXPERIMENTAL

Design of genes for human-like light and heavy chains

The sequence of the human antibody Eu (Sequences of Proteins of Immunological Interest, Kabat, E., et al., U.S. Dept. of Health and Human Services, 1983) was used to provide the framework of the humanized antibody, because the amino acid sequence of the heavy chain of anti-Tac is more homologous to the heavy chain of this antibody than to any other heavy chain sequence in the National Biomedical Foundation Protein Identification Resource.

To select the sequence of the humanized heavy chain, the anti-Tac heavy chain sequence (see, commonly assigned U.S.S.N.'s 186,862 and 223,037, which are incorporated herein by reference) was aligned with the sequence of the Eu heavy chain (Figure 1). At each position, the Eu amino acid was selected for the humanized sequence, unless that position fell in any one of the following categories, in which case the anti-Tac amino acid was selected.

(1) The position fell within a complementarity determining region (CDR), as defined by Kabat, et al., op. cit. (amino acids 31-35, 50-66, 99-106);

(2) The Eu amino acid was unusual for human heavy chains at that position, whereas the anti-Tac amino acid was typical for human heavy chains at that position (amino acids 27, 93, 95, 98, 107-109, 111);

(3) The position was immediately adjacent to a CDR in the amino acid sequence of the anti-Tac heavy chain (amino acids 30 and 67).

(4) 3-dimensional modeling of the anti-Tac antibody suggested that the amino acid was physically close to the antigen binding region (amino acids 48 and 68).

Some amino acids fell in more than one of these categories but are only listed in one.

To select the sequence of the humanized light chain, the anti-Tac light chain sequence was aligned with the sequence of the Eu light chain (Figure 2). The Eu amino acid was selected at each position, unless the position again fell into one of the categories (1) - (4), (with light chain replacing heavy chain in the category definitions):

(1) CDRs (amino acids 24-34, 50-56, 89-97).

(2) Anti-Tac amino acid more typical than Eu (amino acids 48 and 63).

(3) Adjacent to CDRs (no amino acids; Eu and anti-Tac were already the same at all these positions).

(4) Possible 3-dimensional proximity to binding region (amino acid 60).

The actual nucleotide sequence of the heavy (Figure 3) and light chain (Figure 4) genes were selected as follows:

(1) the nucleotide sequences code for the amino acid sequences chosen as described above.

(2) 5' of these coding sequences, the nucleotide sequences code for a leader (signal) sequence, namely the leader of the light chain of the antibody MOPC 63 and the leader of the heavy chain of the antibody PCH 108A (Kabat et al., op. cit.). These leader sequences were chosen as typical of antibodies.

(3) 3' of the coding sequences, the nucleotide sequences are the sequences that follow the mouse light chain J5 segment and the mouse heavy chain J2 segment, which are part of the anti-Tac sequences. These sequences are included because they contain splice donor signals.

(4) At each end of the sequence is an Xba I site to allow cutting at the Xba I sites and cloning into the Xba I site of a vector.

Construction of humanized light and heavy chain genes

To synthesize the heavy chain, four oligonucleotides HES12, HES13, HES14, HES15 (Figure 5A) were synthesized using an Applied Biosystems 380B DNA synthesizer. Two of the oligonucleotides are part of each strand of the heavy chain, and each oligonucleotide overlaps the next one by about 20 nucleotides to allow annealing (Figure 5B). Together, the oligonucleotides cover the entire humanized heavy chain (Figure 3) with a few extra nucleotides at each end to allow cutting at the Xba I sites. The oligonucleotides were purified from polyacrylamide gels.

Each oligonucleotide was phosphorylated using ATP and T4 polynucleotide kinase by standard procedures (see, Maniatis, op. cit.). To anneal the phosphorylated oligonucleotides, they were suspended together in 40 ul of TA (33 mM Tris acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate) at a concentration of about 3.75 uM each, heated to 95 deg for 4 min. and cooled slowly to 4 deg. To synthesize the complete gene from the oligonucleotides by synthesizing the opposite strand of each oligonucleotide (Figure 5B), the following components were added in a final volume of 100ul:

10 ul	annealed oligonucleotides
0.16 mM each	deoxyribonucleotide
0.5 mM	ATP
0.5 mM	DTT
100 ug/ml	BSA
3.5 ug/ml	T4 g43 protein (DNA polymerase)
25 ug/ml	T4 g44/62 protein (polymerase accessory protein)
25 ug/ml	45 protein (polymerase accessory protein)

The mixture was incubated at 37 deg for 30 min. Then 10 u of T4 DNA ligase was added and incubation at 37 deg resumed for 30 min. The polymerase and ligase were inactivated by incubation of the reaction at 70 deg for

15 min. To digest the gene with Xba I, to the reaction was added 50 ul of 2x TA containing BSA at 200 ug/ml and DTT at 1 mM, 43 ul of water, and 50 u of Xba I in 5 ul. The reaction was incubated for 3 hr at 37 deg, and run on a gel. The 431 bp Xba I fragment was purified from a gel and cloned into the Xba I site of the plasmid pUC19 by standard methods. Four plasmid isolates were purified and sequenced using the dideoxy method. One of these had the correct sequence (Figure 3).

To synthesize the light chain, four oligonucleotides JFD1, JFD2, JFD3, JFD4 (Figure 6A) were synthesized. Two of the oligonucleotides are part of each strand of the light chain, and each oligonucleotide overlaps the next one by about 20 nucleotides to allow annealing (Figure 6B). Together, the oligonucleotides cover the entire humanized light chain (Figure 4) with a few extra nucleotides at each end to allow cutting at the Xba I sites. The oligonucleotides were purified from polyacrylamide gels.

The light chain gene was synthesized from these olignucleotides in two parts. 0.5 ug each of JFD1 and JFD2 were combined in 20 ul sequenase buffer (40 mM Tris-HCl, pH 7.5, 20 mM magnesium chloride, 50 mM sodium chloride), heated at 70 deg for 3 min and allowed to cool slowly to 23 deg in order for the oligonucleotides to anneal. JFD3 and JFD4 were treated in the same way. Each reaction was made 10 mM in DTT and 0.5 mM in each deoxyribonucleotide and 6.5 u of sequenase (US Biochemicals) was added, in a final volume of 24 ul, and incubated for 1 hr at 37 deg to synthesize the opposite strands of the oligonucleotides. Xba I and Hind III were added to each reaction to digest the DNA (there is a Hind III site in the region where JFD2 and JFD3 overlap and therefore in each of the synthesized DNAs; Figure 6B). The reactions were run on polyacrylamide gels, and the Xba I - Hind III fragments were purified and cloned into pUC18 by standard methods. Several plasmid isolates for each fragment were sequenced by the dideoxy method, and correct ones chosen.

Construction of plasmids to express humanized light and heavy chains

The heavy chain Xba I fragment was isolated from the pUC19 plasmid in which it had been inserted and then inserted into the Xba I site of the vector pVγ1 (see, commonly assigned U.S.S.N. 223,037) in the correct orientation by standard methods, to produce the plasmid pHuGTAC1 (Figure 7). This plasmid will express high levels of a complete heavy chain when transfected into an appropriate host cell.

The two light chain Xba I - Hind III fragments were isolated from the pUC18 plasmids in which they had been inserted. The vector plasmid pVκ1 (see, commonly assigned U.S.S.N. 223,037) was cut with Xba I, dephosphorylated and ligated with the two fragments by standard methods. The desired reaction product has the circular form: vector - Xba I - fragment 1 - Hind III - fragment 2 - Xba I - vector. Several plasmid isolates were analyzed by restriction mapping and sequencing, and one with this form chosen. This plasmid, pHuLTAC (Figure 8), therefore contains the complete humanized light chain (Figure 4) and will express high levels of the light chain when transfected into an appropriate host cell.

Synthesis and affinity of humanized antibody

The plasmids pHuGTAC1 and pHuLTAC were transfected into mouse Sp2/0 cells, and cells that integrated the plasmids were selected on the basis of resistance to mycophenolic acid and/or hygromycin B conferred by the gpt and hyg genes on the plasmids (Figures 7,8) by standard methods. To verify that these cells secreted antibody that binds to the IL-2 receptor, supernatant from the cells was incubated with HUT-102 cells that are known to express the IL-2 receptor. After washing, the cells were incubated with fluorescein-conjugated goat anti-human antibody, washed, and analyzed for fluorescence on a FACSCAN cytofluorometer. The results (Figure 9A), clearly show that the humanized antibody binds to these cells, but not to Jurkat T-cells that do not express the IL-2 receptor (Figure 9D). As controls, the

original mouse anti-Tac antibody was also used to stain these cells (Figure 9B,C), giving similar results.

For further experiments, cells producing the humanized antibody were injected into mice, and the resultant ascites collected. Humanized antibody was purified to substantial homogeneity from the ascites by passage through an affinity column of goat anti-human immunoglobulin antibody, prepared on an Affigel-10 support (Bio-Rad Laboratories, Inc., Richmond, CA) according to standard techniques. To determine the affinity of the humanized antibody relative to the original anti-Tac antibody, a competitive binding experiment was performed. About 5×10^5 HUT-102 cells were incubated with known quantities (10 - 40 ng) of the anti-Tac antibody and the humanized anti-Tac antibody for 10 min at 4 deg. Then 100 ng of biotinylated anti-Tac was added to the cells and incubated for 30 min at 4 deg. This quantity of anti-Tac had previously been determined to be sufficient to saturate the binding sites on the cells, but not to be in large excess. Then the cells were washed twice with 2 ml of phosphate buffered saline (PBS) containing 0.1% sodium azide. The cells were then incubated for 30 min at 4 deg with 250 ng of phycoerythrin-conjugated avidin, which bound to the biotinylated anti-Tac already bound to the cells. The cells were washed again as above, fixed in PBS containing 1% paraformaldehyde, and analyzed for fluorescence on a FACSCAN cytofluorometer.

Use of increasing amounts (10 - 40 ng) of the anti-Tac antibody as competitor in the first step decreased the amount of biotinylated anti-Tac that could bind to the cells in the second step, and therefore the amount of phycoerythrin-conjugated avidin that bound in the last step, thus decreasing fluorescence (Figure 10A). Equivalent amounts (20 ng) of anti-Tac, and humanized anti-Tac used as competitor decreased the fluorescence to approximately the same degree (Figure 10B). This shows that these antibodies have approximately the same affinity, because if one had greater affinity, it would have more effectively competed