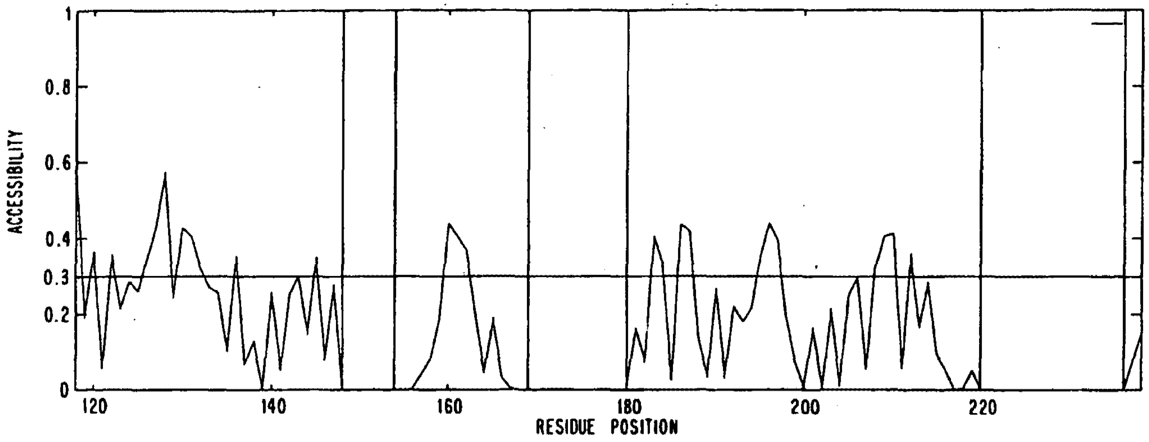
FIG. 3B



```
             HFR 1               CDR H1    HFR 2   CDR H2                          HFR 3                    CDR H3
 | | |   ||| |||   |       | |  *******   |||    ************  || ||       |||          ||| |      *****************

glb2 QVQLQQSGTELARPGASVRLSCKASGYTFTTFGIT--WVKQRTGQGLEWIGEIFPGNS--KTYYAERFKGKATLTADKSSTTAYMQLSSLTSEDSAVYFCAREIR-------------YWG  (SEQ ID NO: 13)
1fdl QVQLKESGPGLVAPSQSLSITCTVSGFSLTGYGVN--WVRQPPGKGLEWLGMIWGDG---NTDYNSALKSRLSISKDNSKSQVFLKMNSLHTDDTARYYCARERDYRL---------DYWG  (SEQ ID NO: 14)
2hfl -VQLQQSGAELMKPGASVKISCKASGYTFSDYWIE--WVKQRPGHGLEWIGEILPGSG--STNYHERFKGKATFTADTSSSTAYMQLNSLTSEDSGVYYCLHGNYDF----------DGWG  (SEQ ID NO: 15)
3hfm DVQLQESGPSLVKPSQTLSLTCSVTGDSITSDYWS--WIRKFPGNRLEYMGYVSYSG---STYYNPSLKSRISITRDTSKNQYYLDLNSVTTEDTATYYCANWDG------------DYWG  (SEQ ID NO: 16)
2fbj EVKLLESGGGLVQPGGSLKLSCAASGFDFSKYWMS--WVRQAPGKGLEWIGEIHPDSG--TINYTPSLKDKFIISRDNAKNSLYLQMSKVRSEDTALYYCARLHYYGYN--------AYWG  (SEQ ID NO: 17)
2fb4 EVQLVQSGGGVVQPGRSLRLSCSSSGFIFSSYAMY--WVRQAPGKGLEWVAIIWDDGS--DQHYADSVKGRFTISRNDSKNTLFLQMDSLRPEDTGVYFCARDGGHGFCSSASCFGPDYWG  (SEQ ID NO: 18)
2mcp EVKLVESGGGLVQPGGSLRLSCATSGFTFSDFYME--WVRQPPGKRLEWIAASRNKGNKYTTEYSASVKGRFIVSRDTSQSILYLQMNALRAEDTAIYYCARNYYGSTWYF------DVWG  (SEQ ID NO: 19)
7fab -VQLEQSGPGLVRPSQTLSLTCTVSGTSFDDYYST--WVRQPPGRGLEWIGYVFYHG---TSDTDTPLRSRVTMLVNTSKNQFSLRLSSVTAADTAVYYCARNLIAGCI--------DVWG  (SEQ ID NO: 20)
4fab EVKLDETGGGLVQPGRPMKLSCVASGFTFSDYWMN--WVRQSPEKGLEWVAQIRNKPYNYETYYSDSVKGRFTISRDDSKSSVYLQMNNLRVEDMGIYYCTGSYYGM----------DYWG  (SEQ ID NO: 21)
1f19 QVQLKESGAELVAASSSVKMSCKASGYTFTSYGVN--WVKQRPGQGLEWIGYINPGKG--YLSYNEKFKGKTTLTVDRSSSTAYMQLRSLTSEDSAVYFCARSFYGGSDLAVYYF--DSWG  (SEQ ID NO: 22)
6fab EVQLQQSGVELVRAGSSVKMSCKASGYTFTSNGIN--WVKQRPGQGLEWIGYNNPGNG--YIAYNEKFKGKTTLTVDKSSSTAYMQLRSLTSEDSAVYFCARSEYYGGSYKF-----DYWG  (SEQ ID NO: 23)
1dfb EVQLVESGGGLVQPGRSLRLSCAASGFTFNDYAMH--WVRQAPGKGLEWVSGISWDSS--SIGYADSVKGRFTISRDNAKNSLYLQMNSLRAEDMALYYCVKGRDYYDSGGYFTVAFDIWG  (SEQ ID NO: 24)
```

FIG. 4A

Light Chain Sequences

```
                                      10        20         30        40         50         60         70
                              ---------+---------+--- ------+---------+ ---------+----- ----+-- -------+
1 N901L                      :DVLMTQTPLSLPVSLGDQASISC RSSQIIIHSDGNTY-LE WFLQKPGQSPKLLIY KVSNRFS GVPDRFSG

2 KOL                        :QSVLTQPPSASG-TPGQRVTISC SGTSSNIGS----STVN WYQQLPGMAPKLLIY RDAMRPS GVPDRFSG
                              | *     |    |  |
3 N901L/KOL                  :QVLMTQTPSSLPVTLGQQASISC RSSQIIIHSDGNTY-LE WFLQKPGQSPKLLIY KVSNRFS GVPDRFSG

4 KV2F$HUMAN                 :DVVMTQSPLSLPVTLGQPASISC RSSQSLVYSDGNTY-LN WFQQRPGQSPRRLIY KVSNRDS GVPDRFSG
  [most identical seq]          *   |      |  ||                                  *
5 N901L/KV2F                 :DVLMTQSPLSLPVTLGQPASISC RSSQIIIHSDGNTY-LE WFQQRPGQSPRLLIY KVSNRFS GVPDRFSG
  [CDR grafted]
6 KV4B$HUMAN                 :DIVMTQSPDSLAVSLGERATINC KSSQSVLYSSNNKNYLA WYQQKPGQPPKLLIY WASTRES GVPDRFSG
  [most identical surf]         *     |        |
7 N901L/KV4B                 :DVLMTQTPDSLPVSLGDRASISC RSSQIIIHSDGNTY-LE WFLQKPGQSPKLLIY KVSNRFS GVPDRFSG
  [Resurfaced]                                       [        L1       ]               [  L2   ]


                                      80        90        100       110
                              ---------+---------+---- -----+----- ----+-----
1 N901L                      :SGSGTDFTLMISRVEAEDLGVYYC FQGSH--VPHT FGGGTKLEI-           (SEQ ID NO: 25)

2 KOL                        :SKSGASASLAIGGLQSEDETDYYC AAWDVSLNAYV FGTGTKVTVL    ( 44)  (SEQ ID NO: 26)
                                   |   |        |                    *
3 N901L/KOL                  :SGSGTSFTLAISRVEAEDEGVYYC FQGSH--VPHT FGGGTKLEI-    (104)  (SEQ ID NO: 27)

4 KV2F$HUMAN                 :SGSGTDFTLKISRVEAEDVGVYYC MQGTH--WSWT FGQGTKVEIK    ( 87)  (SEQ ID NO: 28)
  [most identical seq]                 |        |                    *
5 N901L/KV2F                 :SGSGTDFTLKISRVEAEDVGVYYC FQGSH--VPHT FGGGTKVEI-    (101)  (SEQ ID NO: 29)
  [CDR grafted]
6 KV4B$HUMAN                 :SGSGTDFTLTISSLQAEDVAVYYC QQYDT---IPT FGGGTKVEIK    ( 71)  (SEQ ID NO: 30)
  [most identical surf]
7 N901L/KV4B                 :SGSGTDFTLMISRVEAEDLGVYYC FQGSH--VPHT FGGGTKLEI-    (109)  (SEQ ID NO: 31)
  [Resurfaced]                                          [     L3    ]
```

FIG. 4B

Heavy Chain Sequences

```
                          120       130       140        150        160        170       180
                         --+---------+---------+------- --+---- -----+-------- -+---------+ -------
1 N901H                 :DVQLVESGGGLVQPGGSRKLSCAASGFTFS SFGMH-- WVRQAPEKGLEWVA YISSGSF--TIY HADTVKG

2 KOL                   :EVQLVQSGGGVVQPGRSLRLSCSSSGFIFS SYAMY-- WVRQAPGKGLEWVA IIWDDGS--DQH YADSVKG
                         |         |    | ||        |                  |           ||          |
3 N901H/KOL             :EVQLVESGGGVVQPGRSLRLSCAASGFIFS SFGMH-- WVRQAPGKGLEWVA YISSDGF--TIY HADSVKG

4 G36005                :QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYAMH-- WVRQAPGKGLEWVA VISYDGS--NKY YADSVKG
  [most identical seq]   |         |    | ||                           |                    |  |
5 N901H/G36005          :QVQLVESGGGVVQPGRSLRLSCAASGFTFS SFGMH-- WVRQAPGKGLEWVA YISSGSF--TIY YADSVKG
  [CDR grafted]
6 PL0123                :EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYWMS-- WVRQAPGKGLEWVA NIKQDGS--EKY YVDSVKG
  [most identical surf]  |                ||                           |                       |
7 N901H/PL0123          :EVQLVESGGGLVQPGGSLRLSCAASGFTFS SFGMH-- WVRQAPGKGLEWVA YISSGSF--TIY HADSVKG
  [Resurfaced]                                         [  H1   ]              [     H2     ]


                          190       200       210        220       230        240
                         --+---------+---------+--------- +---------+------ ---+------
1 N901H                 :RFTISRDNPKNTLFLQMTSLRSEDTAMYYCAR MRKGYAM--------DY WGQGTTVTVS           (SEQ ID NO: 32)

2 KOL                   :RFTISRNDSKNTLFLQMDSLRPEDTGVYFCAR DGGHGFCSSASCFGPDY WGQGTPVTVS    ( 77)  (SEQ ID NO: 33)
                                |*            *
3 N901H/KOL             :RFTISRDDPKNTLFLQMTSLRSEDTAMYYCAR MRKGYAM--------DY WGQGTTVTVS    (106)  (SEQ ID NO: 34)

4 G36005                :RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR DRKDWGWALF-----DY WGQGTLVTVS    ( 89)  (SEQ ID NO: 35)
  [most identical seq]           |    |   |   |    |
5 N901H/G36005          :RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR MRKGYAM--------DY WGQGTLVTVS    (103)  (SEQ ID NO: 36)
  [CDR grafted]
6 PL0123                :RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR ----------------- ----------    ( 74)  (SEQ ID NO: 37)
  [most identical surf]
7 N901H/PL0123          :RFTISRDNAKNTLFLQMTSLRAEDTAMYYCAR MRKGYAM--------DY WGQGTTVTVS    (110)  (SEQ ID NO: 38)
  [Resurfaced]                                             [        H3       ]
```

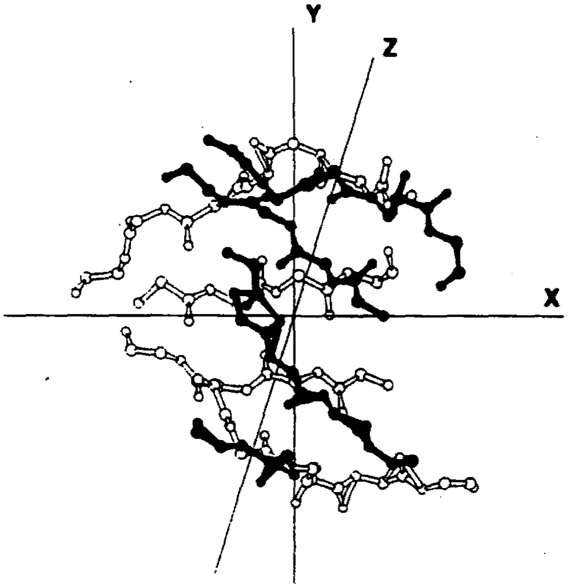
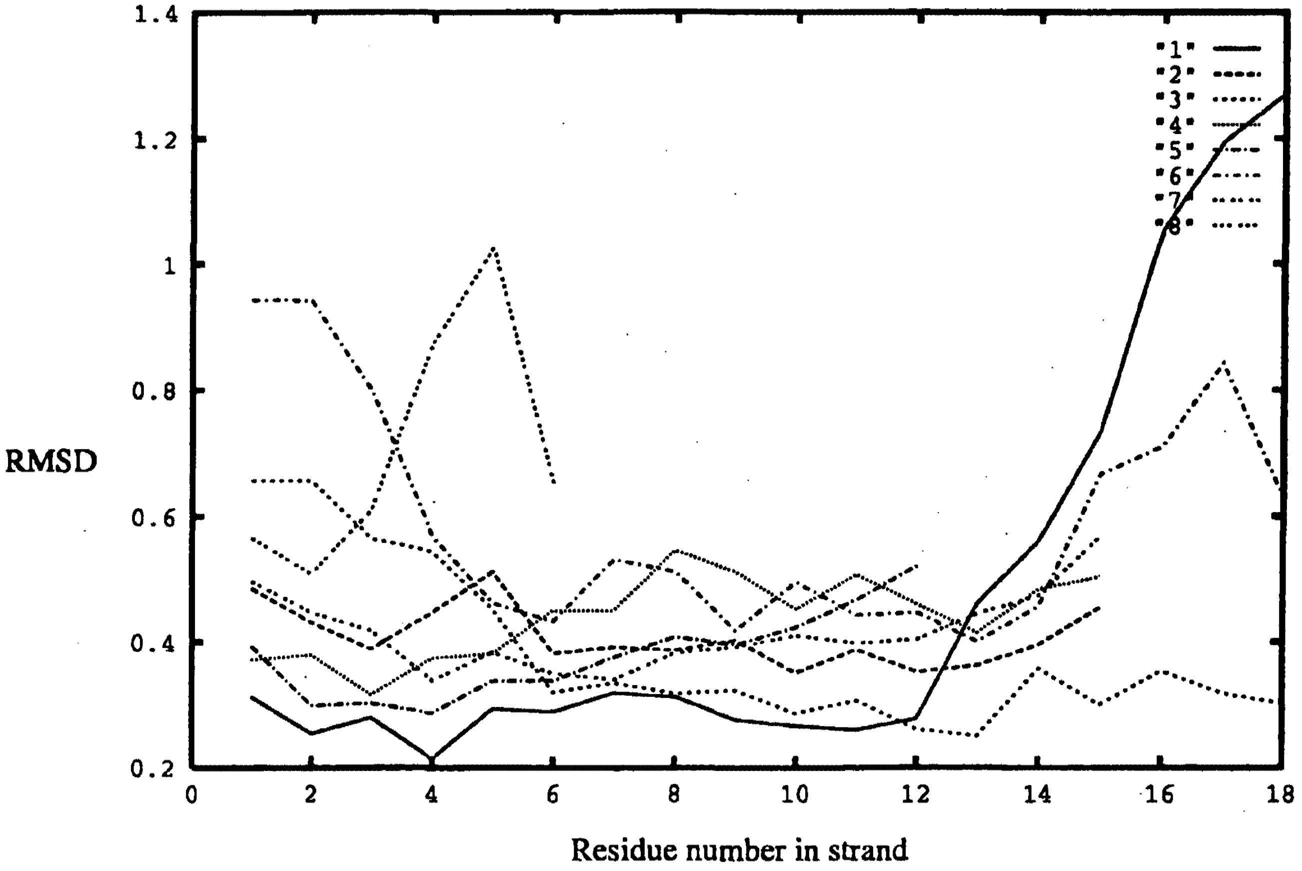


FIG. 5

FIG. 6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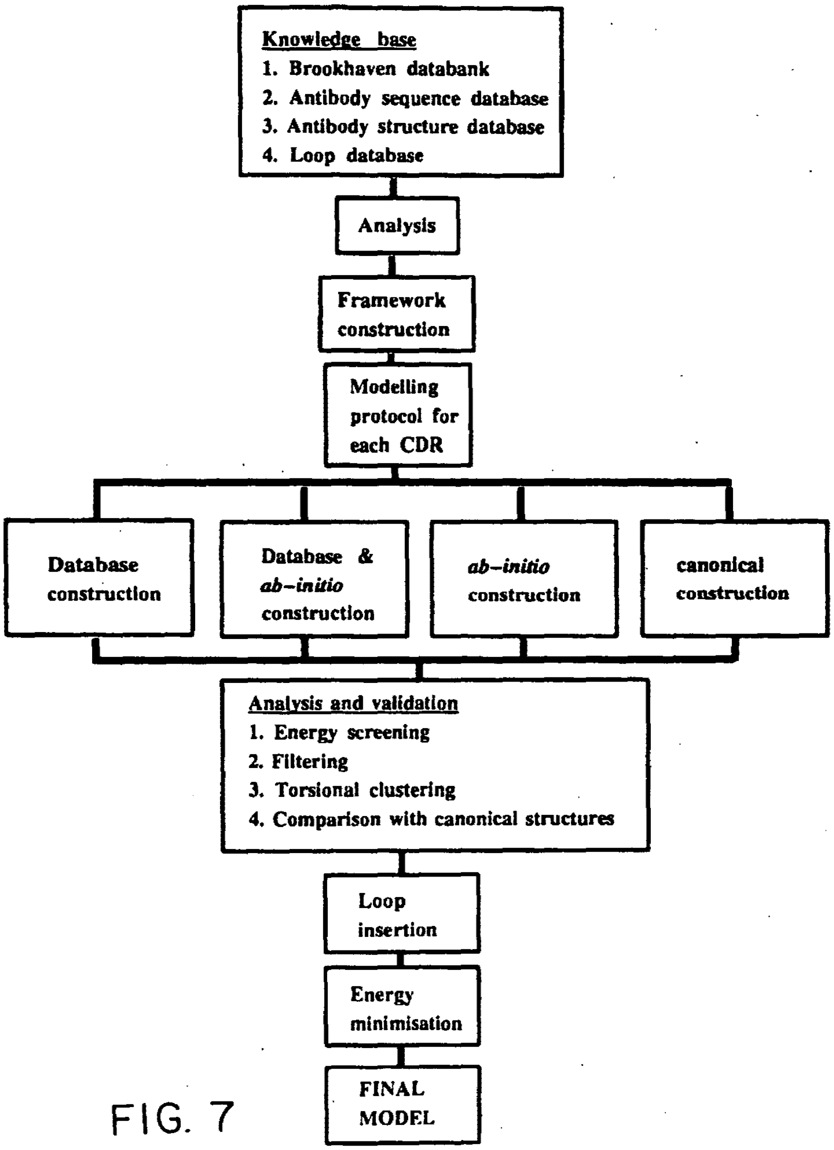

Knowledge base
1. Brookhaven databank
2. Antibody sequence database
3. Antibody structure database
4. Loop database
Analysis
Framework construction
Modelling protocol for each CDR
Database construction
Database & ab-initio construction
ab-initio construction
canonical construction
Analysis and validation
1. Energy screening
2. Filtering
3. Torsional clustering
4. Comparison with canonical structures
Loop insertion
Energy minimisation
FINAL MODEL

FIG. 7

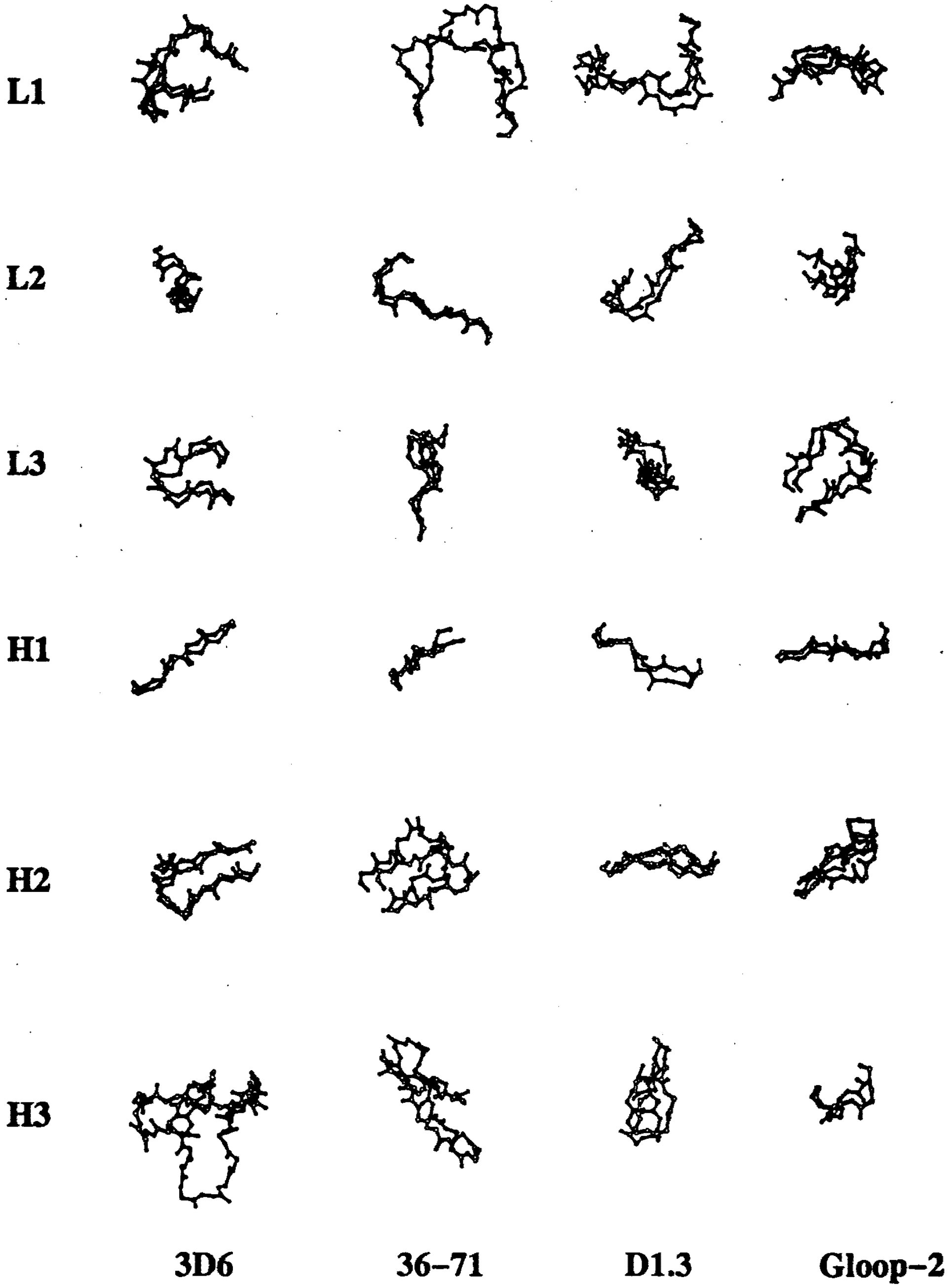


FIG. 8

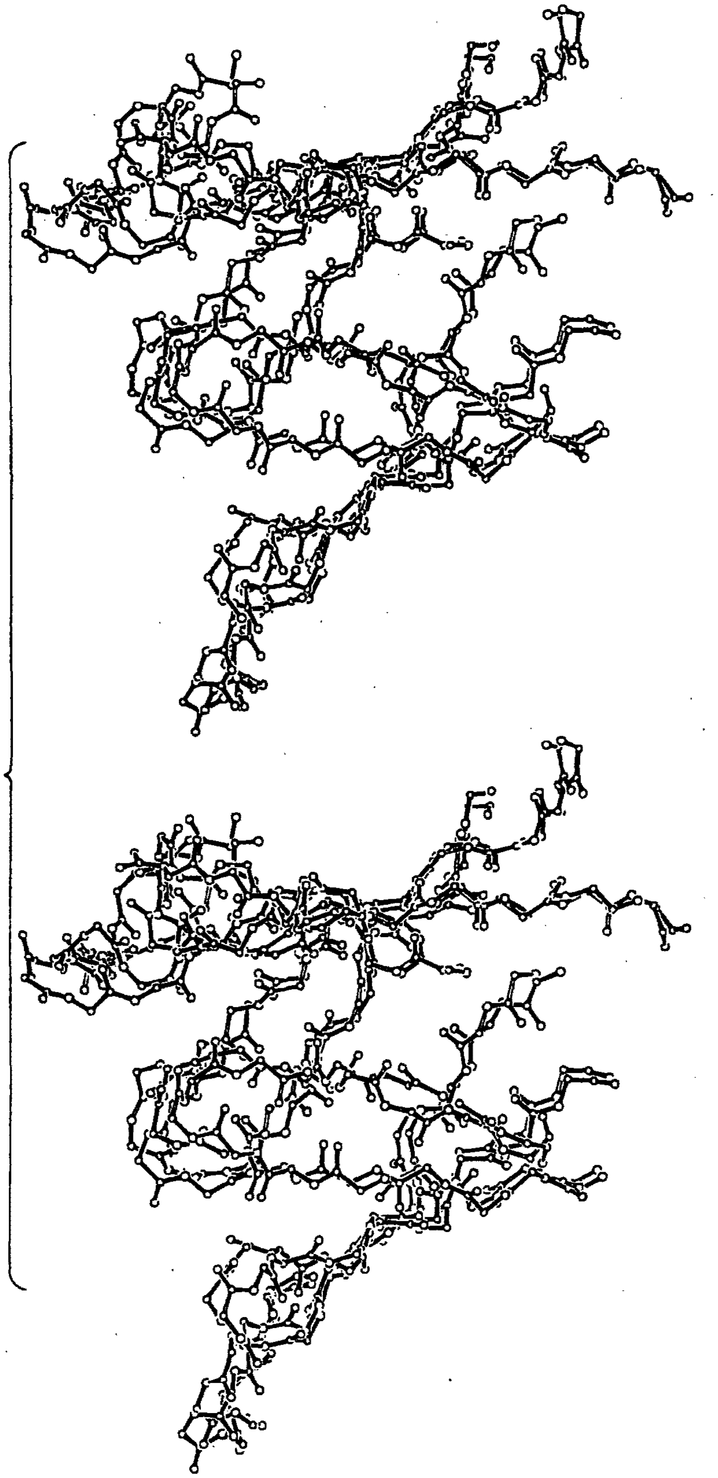
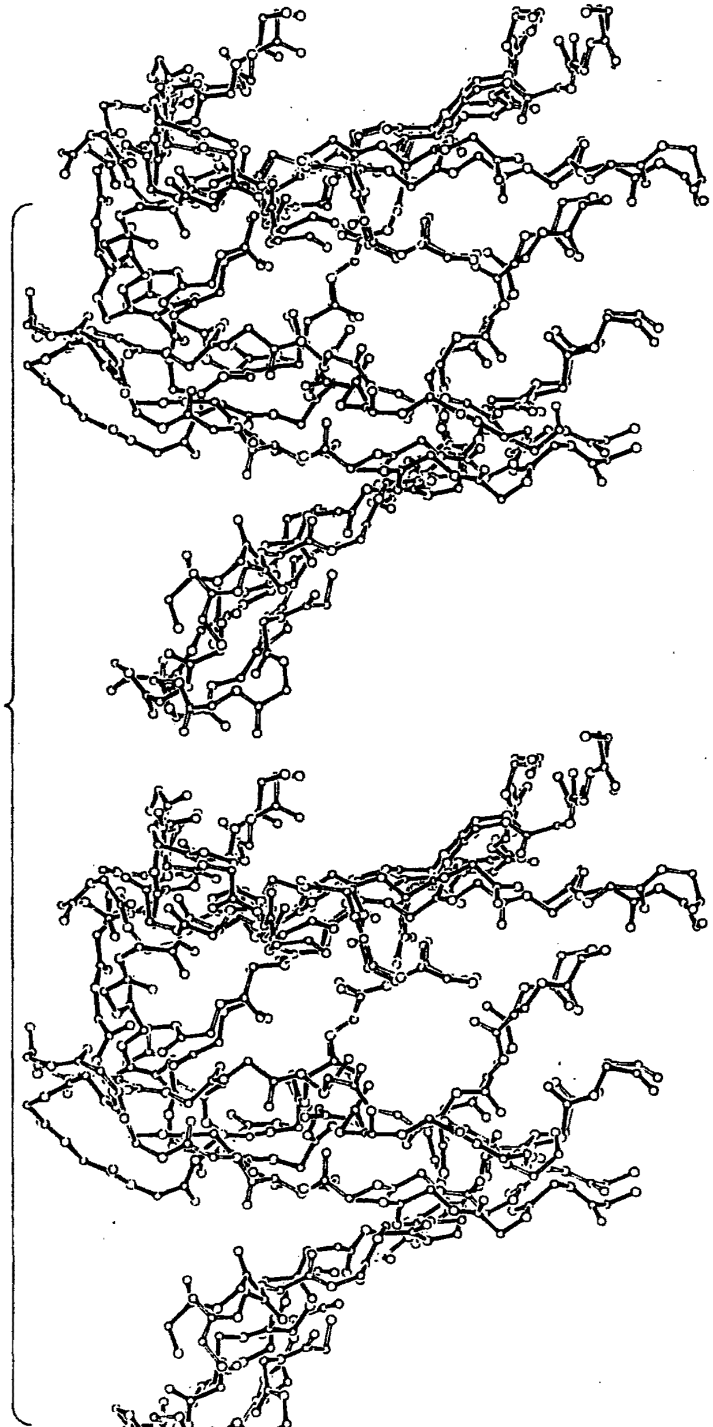


FIG. 9A

FIG. 9B



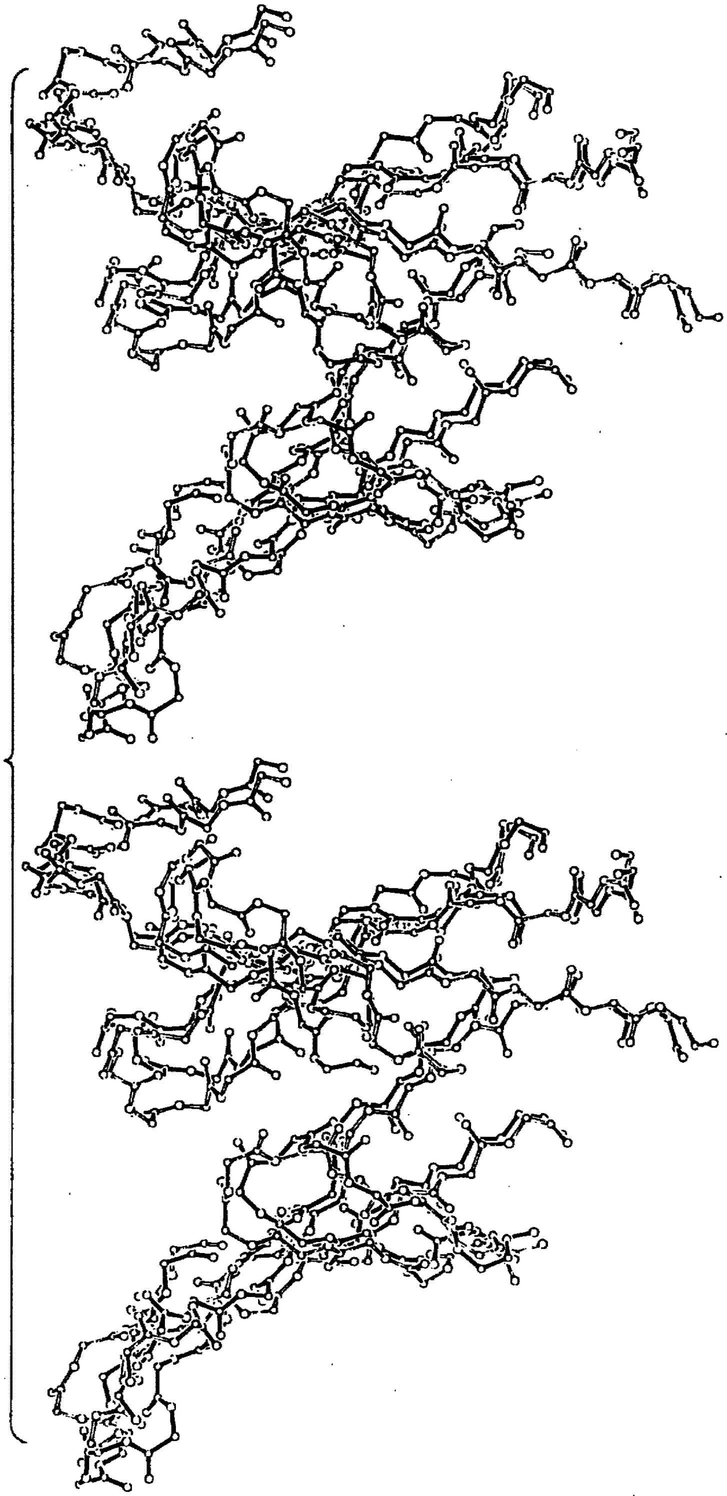
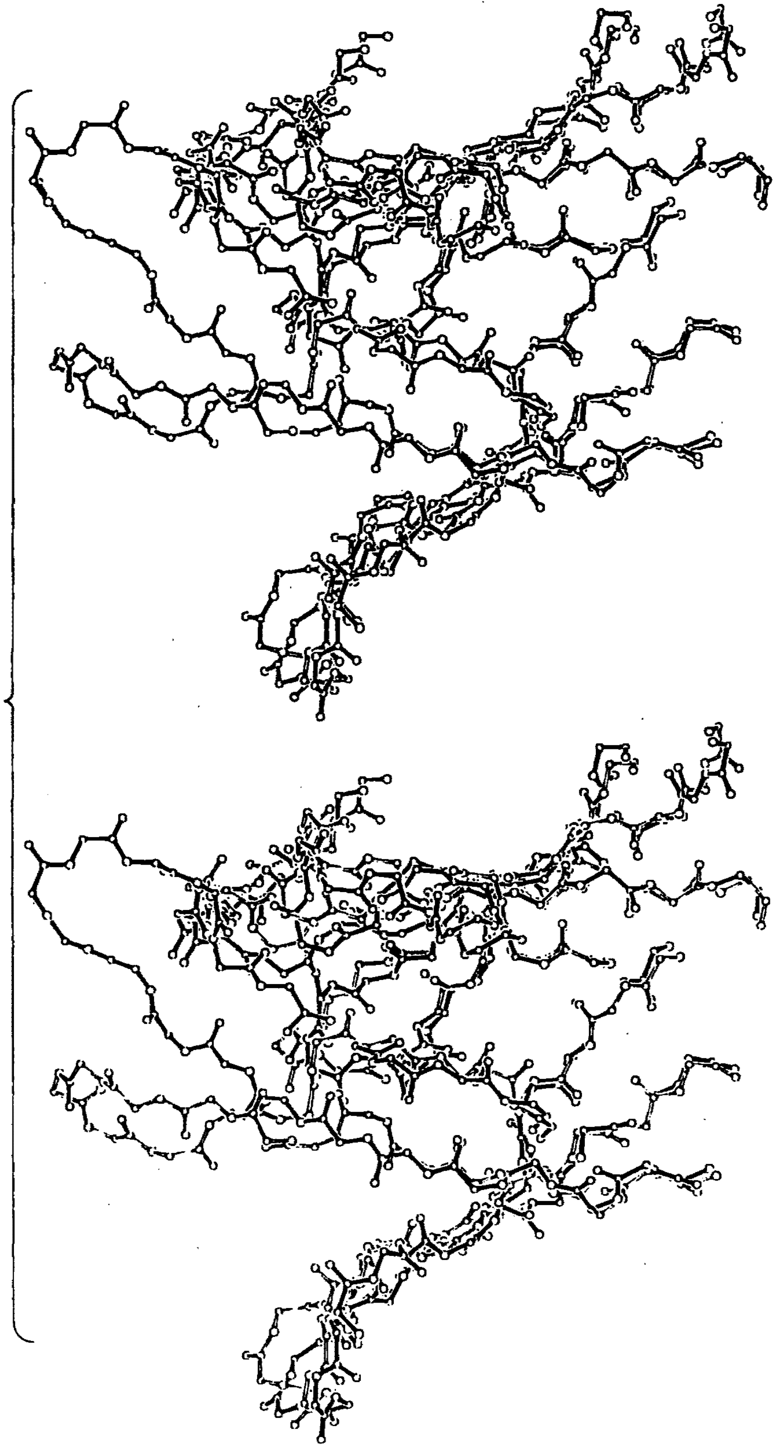
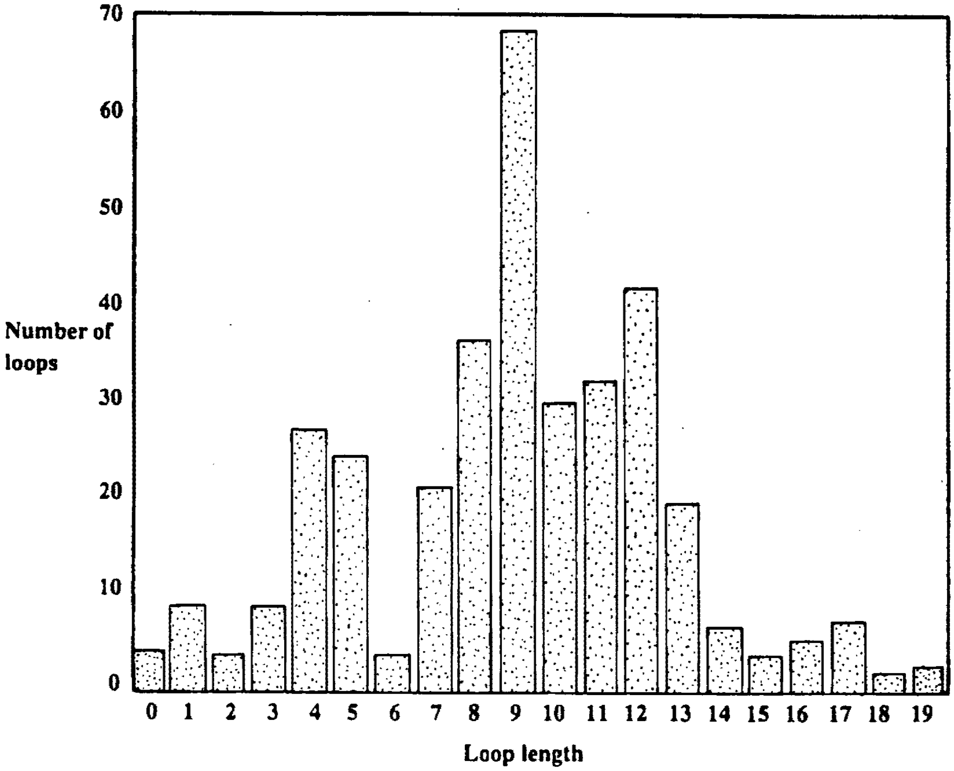


FIG. 9C

FIG. 9D

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12
13
14
15
16
17
18
19
Loop length

FIG. 10

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 93 30 7051

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,A	MOLECULAR IMMUNOLOGY vol. 28, no. 4/5, 1991, GB pages 489 - 498 PADLAN A E 'POSSIBLE PROCEDURE FOR REDUCING THE IMMUNOGENICITY OF ANTIBODY VARIABLE DOMAINS WHILE PRESERVING THEIR LIGAND-BINDING PROPERTIES' * Materials and Methods, p.490, 491, Tables 1-3 *		C12N15/13 C12N15/62 C07K15/00 C12P21/08
D,A	WO-A-9 109 967 (CELLTECH LIMITED) 11 July 1991 * p. 5, second paragraph, p. 6 second paragraph, "Rational" pp. 19-23 *		
P,A	EP-A-0 519 596 (MERCK & CO. INC.) 23 December 1992 * Claims *		
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C07K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	12 JANUARY 1994	Germinario C.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)

⑲ Europäisches Patentamt
European Patent Office
Office européen des brevets

⑪ Publication number: **0 620 276 A1**

⑫ EUROPEAN PATENT APPLICATION

㉑ Application number: **94104042.0**

㉒ Date of filing: **21.12.90**

(51) Int. Cl.5: **C12N 15/13**, C07K 15/28, A61K 39/395, G01N 33/577

This application was filed on 16 - 03 - 1994 as a divisional application to the application mentioned under INID code 60.

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

㉚ Priority: **21.12.89 GB 8928874**

㊸ Date of publication of application: **19.10.94 Bulletin 94/42**

(60) Publication number of the earlier application in accordance with Art.76 EPC: **0 460 167**

(84) Designated Contracting States: **AT BE CH DE DK ES FR GB GR IT LI LU NL SE**

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(54) Humanised antibodies.

(57) CDR-grafted antibody heavy and light chains comprise acceptor framework and donor antigen binding regions, the heavy chains comprising donor residues at at least one of positions (6, 23) and/or (24, 48) and/or (49, 71) and/or (73, 75) and/or (76) and/or (78) and/or (91). The CDR-grated light chains comprise donor residues at at least one of positions (1) and/or (3) and (46) and/or (47) or at at least one of positions (46, 48, 58) and (71). The CDR-grafted antibodies are preferably humanised antibodies, having non-human, e.g. rodent, donor residues and human acceptor frameworks, and may be used for *in vivo* therapy and diagnosis. A generally applicable protocol is disclosed for obtaining CDR-grafted antibodies.

EP 0 620 276 A1

Rank Xerox (UK) Business Services
(3.10/3.09/3.3.4)

```
  1  GAATTCCCAA AGACAAAatg gattttcaag tgcagatttt cagcttcctg
 51  ctaatcagtg cctcagtcat aatatccaga ggacaaattg ttctcaccca
101  gtctccagca atcatgtctg catctccagg ggagaaggtc accatgacct
151  gcagtgccag ctcaagtgta agttacatga actggtacca gcagaagtca
201  ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
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501  cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
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651  agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
701  gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
751  CCAGCTCCCA GCTCCATCCT ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
801  CCACAAGCGC tTACCACTGT TGCGGTGCTC tAAACCTCCT CCCACCTCCT
851  TCTCCTCCTC CTCCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
901  AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA
```

Fig. 1(a)

```
  1  MDFQVQIFSF LLISASVIIS RGQIVLTQSP AIMSASPGEK VTMTCSASSS
 51  VSYMNWYQQK SGTSPKRWIY DTSKLASGVP AHFRGSGSGT SYSLTISGME
101  AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
151  ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
201  TLTKDEYERH NSYTCEATHK TSTSPIVKSF NRNEC*
```

Fig. 1(b)

Field of the Invention

The present invention relates to humanised antibody molecules, to processes for their production using recombinant DNA technology, and to their therapeutic uses.

The term "humanised antibody molecule" in used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site typically comprises complementarity determining regions (CDRs) which determine the binding specificity of the antibody molecule and which are carried on appropriate framework regions in the variable domains. There are 3 CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Background of the Invention

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, (Fab')$_2$ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, were hindered until recently by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies (MAbs) of defined specificity (1).

However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness. In practice, MAbs of rodent origin may not be used in patients for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. For instance, OKT3 a mouse IgG2a/k MAb which recognises an antigen in the T-cell receptor-CD3 complex has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection [Chatenoud et al (2) and Jeffers et al (3)-]. However, in view of the rodent nature of this and other such MAbs, a significant HAMA response which may include a major anti-idiotype component, may build up on use. Clearly, it would be highly desirable to diminish or abolish this undesirable HAMA response and thus enlarge the areas of use of these very useful antibodies.

Proposals have therefore been made to render non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These techniques typically involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MAbs involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP0120694 (Celltech Limited), EP0125023 (Genentech Inc. and City of Hope), EP-A-0 171496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), and WO 86/01533 (Celltech Limited). This latter Celltech application (WO 86/01533) discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. Such humanized chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period [Begent et al (ref. 4)].

In an alternative approach, described in EP-A-0239400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The present invention relates to humanized antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanized antibodies are much less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the much lower proportion of non-human amino acid sequence which they contain.

The earliest work on humanizing MAbs by CDR-grafting was carried out on MAbs recognizing synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAb recognizing lysozyme and a rat MAb recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen et al (5) and Riechmann et al (6) respectively. The preparation of CDR-grafted antibody to the antigen on human T cells is also described in WO 89/07452 (Medical Research Council).

In Riechmann et al/Medical Research Council it was found that transfer of the CDR regions alone [as defined by Kabat refs. (7) and (8)] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having improved antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanised antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, in particular in the structural loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognise more complex antigens. Even so the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAb.

Very recently Queen et al (9) have described the preparation of a humanised antibody that binds to the interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. The human framework regions were chosen to maximise homology with the anti-Tac MAb sequence. In addition computer modelling was used to identify framework amino acid residues which wore likely to interact with the CDRs or antigen, and mouse amino acids were used at these positions in the humanised antibody.

In WO 90/07861 Queen et al propose four criteria for designing humanised immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is unusually homologous to the non-human donor immunoglobulin to be humanised, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanised immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, and may be applied singly or in any combination.

WO 90/07861 describes in detail the preparation of a single CDR-grafted humanised antibody, a humanised antibody having specificity for the p55 Tac protein of the IL-2 receptor. The combination of all four criteria, as above, were employed in designing this humanized antibody, the variable region frameworks of the human antibody Eu (7) being used as acceptor. In the resultant humanised antibody the donor CDRs were as defined by Kabat et al (7 and 8) and in addition the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in the heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanised anti-Tac antibody obtained is reported to have an affinity for p55 of 3 x 10^9 M^{-1}, about one-third of that of the murine MAb.

We have further investigated the preparation of CDR-grafted humanised antibody molecules and have identified a hierarchy of positions within the framework of the variable regions (i.e. outside both the Kabat CDRs and structural loops of the variable regions) at which the amino acid identities of the residues are important for obtaining CDR-grafted products with satisfactory binding affinity. This has enabled us to establish a protocol for obtaining satisfactory CDR-grafted products which may be applied very widely irrespective of the level of homology between the donor immunoglobulin and acceptor framework. The set of residues which we have identified as being of critical importance does not coincide with the residues identified by Queen et al (9).

Summary of the Invention

Accordingly, in a first aspect the invention provides a CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

In preferred embodiments, the heavy chain framework comprises donor residues at positions 23, 24, 49, 71, 73 and 78 or at positions 23, 24 and 49. The residues at positions 71, 73 and 78 of the heavy chain framework are preferably either all acceptor or all donor residues.

In particularly preferred embodiments the heavy chain framework additionally comprises donor residues at one, some or all of positions 6, 37, 48 and 94. Also it is particularly preferred that residues at positions of the heavy chain framework which are commonly conserved across species, i.e. positions 2, 4, 25, 36, 39, 47, 93, 103, 104, 106 and 107, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the heavy chain framework additionally comprises donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

In addition the heavy chain framework optionally comprises donor residues at one, some or all of positions:
1 and 3,
72 and 76,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
80 and 20 (if 69 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91,
88, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

In the first and other aspects of the present invention reference is made to CDR-grafted antibody products comprising acceptor framework and donor antigen binding regions. It will be appreciated that the invention is widely applicable to the CDR-grafting of antibodies in general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

In the first and other aspects of the present invention, the donor antigen binding region typically comprises at least one CDR from the donor antibody. Usually the donor antigen binding region comprises at least two and preferably all three CDRs of each of the heavy chain and/or light chain variable regions. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a composite of the Kabat and structural loop CDRs and any combination of any of these. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR2 (residues 50-65) and CDR3 (residues 95-100) and a composite of the Kabat and structural loop CDRs at CDR1 (residues 26-35).

The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering [refs. (7) and (8)]. Thus the residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. For example, the heavy chain variable region of the anti-Tac antibody described by Queen et al (9) contains a single amino acid insert (residue 52a) after residue 52 of CDR2 and a three amino acid insert (residues 82a, 82b and 82c) after framework residue 82, in the Kabat numbering. The correct Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The invention also provides in a second aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47. Preferably the CDR grafted light chain of the second aspect comprises donor residues at positions 46 and/or 47.

The invention also provides in a third aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

In a preferred embodiment of the third aspect, the framework comprises donor residues at all of positions 46, 48, 58 and 71.

In particularly preferred embodiments of the second and third aspects, the framework additionally comprises donor residues at positions 36, 44, 47, 85 and 87. Similarly positions of the light chain framework which are commonly conserved across species, i.e. positions 2, 4, 6, 35, 49, 62, 64-69, 98, 99, 101 and 102, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the light chain framework additionally comprises donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

In addition the framework of the second or third aspects optionally comprises donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form at potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 is different between donor and acceptor),
and
any one or more of 10, 12, 40, 80, 103 and 105.

Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR1 (residue 24-34), CDR2 (residues 50-56) and CDR3 (residues 89-97).

The invention further provides in a fourth aspect a CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain and at least one CDR-grafted light chain according to the first and second or first and third aspects of the invention.

The humanised antibody molecules and chains of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, (Fab')2 or FV fragment; a light chain or heavy chain monomer or dimer; or a single chain antibody, e.g. a single chain FV in which heavy and light chain variable regions are joined by a peptide linker; or any other CDR-grafted molecule with the same specificity as the original donor antibody. Similarly the CDR-grafted heavy and light chain variable region may be combined with other antibody domains as appropriate.

Also the heavy or light chains or humanised antibody molecules of the present invention may have attached to them an effector or reporter molecule. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce an immunoglobulin molecule in which the Fc fragment or CH3 domain of a complete immunoglobulin molecule has been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

Any appropriate acceptor variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived. Preferably, the type of acceptor framework used is of the same/similar class/type as the donor antibody. Conveniently, the framework may be chosen to maximise/optimise homology with the donor antibody sequence particularly at positions close or adjacent to the CDRs. However, a high level of homology between donor and acceptor sequences is not important for application of the present invention. The present invention identifies a hierarchy of framework residue positions at which donor residues may be important or desirable for obtaining a CDR-grafted antibody product having satisfactory binding properties. The CDR-grafted products usually have binding affinities of at least 10^5 M^{-1}, preferably at least about 10^8 M^{-1}, or especially in the range 10^8-10^{12} M^{-1}. In principle, the present invention is applicable to any combination of donor and acceptor antibodies irrespective of the level of homology between their sequences. A protocol for applying the invention to any particular donor-acceptor antibody pair is given hereinafter. Examples of human frameworks which may be used are KOL, NEWM, REI, EU, LAY and POM (refs. 4 and 5) and the like; for instance KOL and NEWM for the heavy chain and REI for the light chain and EU, LAY and POM for both the heavy chain and the light chain.

Also the constant region domains of the products of the invention may be selected having regard to the proposed function of the antibody in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgE, IgG or IgM domains. In particular, IgG human constant region domains may be used, especially of the IgG1 and IgG3 isotypes, when the humanised antibody molecule is intended for therapeutic uses, and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the humanised antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simple blocking of lymphokine activity.

However, the remainder of the antibody molecules need not comprise only protein sequences from immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a functional polypeptide such as an effector or reporter molecule.

Preferably the CDR-grafted antibody heavy and light chain and antibody molecule products are produced by recombinant DNA technology.

Thus in further aspects the invention also includes DNA sequences coding for the CDR-grafted heavy and light chains, cloning and expression vectors containing the DNA sequences, host cells transformed with the DNA sequences and processes for producing the CDR-grafted chains and antibody molecules comprising expressing the DNA sequences in the transformed host cells.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 10 and 11.

The DNA sequences which encode the donor amino acid sequence may be obtained by methods well known in the art. For example the donor coding sequences may be obtained by genomic cloning, or cDNA cloning from suitable hybridoma cell lines. Positive clones may be screened using appropriate probes for the heavy and light chain genes in question. Also PCR cloning may be used.

DNA coding for acceptor, e.g. human acceptor, sequences may be obtained in any appropriate way. For example DNA sequences coding for preferred human acceptor frameworks such as KOL, REI, EU and NEWM, are widely available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example oligonucleotide directed synthesis as described by Jones et al (ref. 20) may be used. Also oligonucleotide directed mutagenesis of a pre-exising variable region as, for example, described by Verhoeyen et al (ref. 5) or Riechmann et al (ref. 6) may be used. Also enzymatic filling in of gapped oligonucleotides using T_4 DNA polymerase as, for example, described by Queen et al (ref. 9) may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial e.g. E. coli, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and $(Fab')_2$ fragments, and especially FV fragments and single chain antibody fragments e.g. single chain FVs. Eucaryotic e.g. mammalian host cell expression systems may be used for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Thus, in a further aspect the present invention provides a process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to the first aspect of the invention;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to the second or third aspect of the invention;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

The CDR-grafted product may comprise only heavy or light chain derived polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence is used to transfect the host cells. For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, the first vector may contain an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical, except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially, genomic DNA, preferably a fusion of cDNA and genomic DNA.

The present invention is applicable to antibodies of any appropriate specificity. Advantageously, however, the invention may be applied to the humanisation of non-human antibodies which are used for in vivo therapy or diagnosis. Thus the antibodies may be site-specific antibodies such as tumour-specific or cell surface-specific antibodies, suitable for use in in vivo therapy or diagnosis, e.g. tumour imaging. Examples of cell surface-specific antibodies are anti-T cell antibodies, such as anti-CD3, and CD4 and adhesion molecules, such as CR3, ICAM and ELAM. The antibodies may have specificity for interleukins (including lymphokines, growth factors and stimulating factors), hormones and other biologically active compounds, and receptors for any of these. For example, the antibodies may have specificity for any of the following: Interferons α, β, γ or δ, IL1, IL2, IL3, or IL4, etc., TNF, GCSF, GMCSF, EPO, hGH, or insulin, etc.

The the present invention also includes therapeutic and diagnostic compositions comprising the CDR-grafted products of the invention and uses of such compositions in therapy and diagnosis.

Accordingly in a further aspect the invention provides a therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Accordingly also the invention provides a method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention to a human or animal subject.

A preferred protocol for obtaining CDR-grafted antibody heavy and light chains in accordance with the present invention is set out below together with the rationale by which we have derived this protocol. This protocol and rationale are given without prejudice to the generality of the invention as hereinbefore described and defined.

Protocol

It is first of all necessary to sequence the DNA coding for the heavy and light chain variable regions of the donor antibody, to determine their amino acid sequences. It is also necessary to choose appropriate acceptor heavy and light chain variable regions, of known amino acid sequence. The CDR-grafted chain is then designed starting from the basis of the acceptor sequence. It will be appreciated that in some cases the donor and acceptor amino acid residues may be identical at a particular position and thus no change of acceptor framework residue is required.

1. As a first step donor residues are substituted for acceptor residues in the CDRs. For this purpose the CDRs are preferably defined as follows:

Heavy chain	- CDR1: residues 26-35
	- CDR2: residues 50-65
	- CDR3: residues 95-102
Light chain	- CDR1: residues 24-34
	- CDR2: residues 50-56
	- CDR3: residues 89-97

The positions at which donor residues are to be substituted for acceptor in the framework are then chosen as follows, first of all with respect to the heavy chain and subsequently with respect to the light chain.

2. Heavy Chain
   2.1 Choose donor residues at all of positions 23, 24, 49, 71, 73 and 78 of the heavy chain or all of positions 23, 24 and 49 (71, 73 and 78 are always either all donor or all acceptor).
   2.2 Check that the following have the same amino acid in donor and acceptor sequences, and if not preferably choose the donor: 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.
   2.3 To further optimise affinity consider choosing donor residues at one, some or any of:
      i. 1, 3
      ii. 72, 76
      iii. If 48 is different between donor and acceptor sequences, consider 69
      iv. If at 48 the donor residue is chosen, consider 38 and 46
      v. If at 69 the donor residue is chosen, consider 80 and then 20
      vi. 67
      vii. If at 67 the donor residue is chosen, consider 82 and then 18
      viii. 91
      ix. 88
      x. 9, 11, 41, 87, 108, 110, 112
3. Light Chain

3.1 Choose donor at 46, 48, 58 and 71

3.2 Check that the following have the same amino acid in donor and acceptor sequences, if not preferably choose donor:

2, 4, 6, 35, 38, 44, 47, 49, 62, 64-69 inclusive, 85, 87, 98, 99, 101 and 102

3.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 63
iii. 60, if 60 and 54 are able to form potential saltbridge
iv. 70, if 70 and 24 are able to form potential saltbridge
v. 73, and 21 if 47 is different between donor and acceptor
vi. 37, and 45 if 47 is different between donor and acceptor
vii. 10, 12, 40, 80, 103, 105

Rationale

In order to transfer the binding site of an antibody into a different acceptor framework, a number of factors need to be considered.

1. The extent of the CDRs

The CDRs (Complementary Determining Regions) were defined by Wu and Kabat (refs. 4 and 5) on the basis of an analysis of the variability of different regions of antibody variable regions. Three regions per domain were recognised. In the light chain the sequences are 24-34, 50-56, 89-97 (numbering according to Kabat (ref. 4), Eu Index) inclusive and in the heavy chain the sequences are 31-35, 50-65 and 95-102 inclusive.

When antibody structures became available it became apparent that these CDR regions corresponded in the main to loop regions which extended from the β barrel framework of the light and heavy variable domains. For H1 there was a discrepancy in that the loop was from 26 to 32 inclusive and for H2 the loop was 52 to 56 and for L2 from 50 to 53. However, with the exception of H1 the CDR regions encompassed the loop regions and extended into the β strand frameworks. In H1 residue 26 tends to be a serine and 27 a phenylalanine or tyrosine, residue 29 is a phenylalanine in most cases. Residues 28 and 30 which are surface residues exposed to solvent might be involved in antigen-binding. A prudent definition of the H1 CDR therefore would include residues 26-35 to include both the loop region and the hypervariable residues 33-35.

It is of interest to note the example of Riechmann et al (ref. 3), who used the residue 31-35 choice for CDR-H1. In order to produce efficient antigen binding, residue 27 also needed to be recruited from the donor (rat) antibody.

2. Non-CDR residues which contribute to antigen binding

By examination of available X-ray structures we have identified a number of residues which may have an effect on net antigen binding and which can be demonstrated by experiment. These residues can be sub-divided into a number of groups.

2.1 Surface residues near CDR [all numbering as in Kabat et al (ref. 7)].

2.1.1. Heavy Chain - Key residues are 23, 71 and 73. Other residues which may contribute to a lesser extent are 1, 3 and 76. Finally 25 is usually conserved but the murine residue should be used if there is a difference.

2.1.2 Light Chain - Many residues close to the CDRs, e.g. 63, 65, 67 and 69 are conserved. If conserved none of the surface residues in the light chain are likely to have a major effect. However, if the murine residue at these positions is unusual, then it would be of benefit to analyse the likely contribution more closely. Other residues which may also contribute to binding are 1 and 3, and also 60 and 70 if the residues at these positions and at 54 and 24 respectively are potentially able to form a salt bridge i.e. 60 + 54; 70 + 24.

2.2 Packing residues near the CDRs.

2.2.1. Heavy Chain - Key residues are 24, 49 and 78. Other key residues would be 36 if not a tryptophan, 94 if not an arginine, 104 and 106 if not glycines and 107 if not a threonine. Residues which may make a further contribution to stable packing of the heavy chain and hence improved affinity are 2, 4, 6, 38, 46, 67 and 69. 67 packs against the CDR residue 63 and this pair could be either both mouse or both human. Finally, residues which contribute to packing in this region but from a longer range are 18, 20, 80, 82 and 86. 82 packs against 67 and in turn 18 packs against 82. 80 packs against 69 and in turn 20 packs against 80. 86 forms an H bond network with 38 and 46. Many of the mouse-human differences appear minor e.g. Leu-Ile, but could have an minor impact on correct packing which could translate into altered positioning of the CDRs.

2.2.2. Light Chain - Key residues are 48, 58 and 71. Other key residues would be 6 if not glutamine, 35 if not tryptophan, 62 if not phenylalanine or tryosine, 64, 66, 68, 99 and 101 if not glycines and 102 if not a threonine. Residues which make a further contribution are 2, 4, 37, 45 and 47. Finally residues 73 and 21 and 19 may make long distance packing contributions of a minor nature.

2.3. Residues at the variable domain interface between heavy and light chains - In both the light and heavy chains most of the non-CDR interface residues are conserved. If a conserved residue is replaced by a residue of different character, e.g. size or charge, it should be considered for retention as the murine residue.

2.3.1. Heavy Chain - Residues which need to be considered are 37 if the residue is not a valine but is of larger side chain volume or has a charge or polarity. Other residues are 39 if not a glutamine, 45 if not a leucine, 47 if not a tryptophan, 91 if not a phenylalanine or tyrosine, 93 if not an alanine and 103 if not a tryptophan. Residue 89 is also at the interface but is not in a position where the side chain could be of great impact.

2.3.2. Light Chain - Residues which need to be considered are 36, if not a tyrosine, 38 if not a glutamine, 44 if not a proline, 46, 49 if not a tyrosine, residue 85, residue 87 if not a tyrosine and 98 if not a phenylalanine.

2.4. Variable-Constant region interface - The elbow angle between variable and constant regions may be affected by alterations in packing of key residues in the variable region against the constant region which may affect the position of V_L and V_H with respect to one another. Therefore it is worth noting the residues likely to be in contact with the constant region. In the heavy chain the surface residues potentially in contact with the variable region are conserved between mouse and human antibodies therefore the variable region contact residues may influence the V-C interaction. In the light chain the amino acids found at a number of the constant region contact points vary, and the V & C regions are not in such close proximity as the heavy chain. Therefore the influences of the light chain V-C interface may be minor.

2.4.1. Heavy Chain - Contact residues are 7, 11, 41, 87, 108, 110, 112.

2.4.2. Light Chain - In the light chain potentially contacting residues are 10, 12, 40, 80, 83, 103 and 105.

The above analysis coupled with our considerable practical experimental experience in the CDR-grafting of a number of different antibodies have lead us to the protocol given above.

The present invention is now described, by way of example only, with reference to the accompanying Figures 1 - 13.

Brief Description of the Figures

Figure 1	shows DNA and amino acid sequences of the OKT3 light chain;
Figure 2	shows DNA and amino acid sequences of the OKT3 heavy chain;
Figure 3	shows the alignment of the OKT3 light variable region amino acid sequence with that of the light variable region of the human antibody REI;
Figure 4	shows the alignment of the OKT3 heavy variable region amino acid sequence with that of the heavy variable region of the human antibody KOL;
Figure 5	shows the heavy variable region amino acid sequences of OKT3, KOL and various corresponding CDR grafts;
Figure 6	shows the light variable region amino acid sequences of OKT3, REI and various corresponding CDR grafts;
Figure 7	shows a graph of binding assay results for various grafted OKT3 antibodies'
Figure 8	shows a graph of blocking assay results for various grafted OKT3 antibodies;
Figure 9	shows a similar graph of blocking assay results;
Figure 10	shows similar graphs for both binding assay and blocking assay results;
Figure 11	shows further similar graphs for both binding assay and blocking assay results;
Figure 12	shows a graph of competition assay results for a minimally grafted OKT3 antibody compared with the OKT3 murine reference standard, and
Figure 13	shows a similar graph of competition assay results comparing a fully grafted OKT3 antibody with the murine reference standard.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

EXAMPLE 1

CDR-GRAFTING OF OKT3

MATERIAL AND METHODS

1. INCOMING CELLS
Hybridoma cells producing antibody OKT3 were provided by Ortho (seedlot 4882.1) and were grown up in antibiotic free Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine and 5% foetal calf serum, and divided to provide both an overgrown supernatant for evaluation and cells for extraction of RNA. The overgrown supernatant was shown to contain 250 ug/mL murine IgG2a/kappa antibody. The supernatant was negative for murine lambda light chain and IgG1, IgG2b, IgG3, IgA and IgM heavy chain. 20mL of supernatant was assayed to confirm that the antibody present was OKT3.

2. MOLECULAR BIOLOGY PROCEDURES
Basic molecular biology procedures were as described in Maniatis et al (ref. 9) with, in some cases, minor modifications. DNA sequencing was performed as described in Sanger et al (ref. 11) and the Amersham International Plc sequencing handbook. Site directed mutagenesis was as described in Kramer et al (ref. 12) and the Anglian Biotechnology Ltd. handbook. COS cell expression and metabolic labelling studies were as described in Whittle et al (ref. 13)

3. RESEARCH ASSAYS

3.1. ASSEMBLY ASSAYS Assembly assays were performed on supernatants from transfected COS cells to determine the amount of intact IgG present.

3.1.1. COS CELLS TRANSFECTED WITH MOUSE OKT3 GENES The assembly assay for intact mouse IgG in COS cell supernatants was an ELISA with the following format:
96 well microtitre plates were coated with F(ab')2 goat anti-mouse IgG Fc. The plates were washed in water and samples added for 1 hour at room temperature. The plates were washed and F(ab')2 goat anti-mouse IgG F(ab')2 (HRPO conjugated) was then added. Substrate was added to reveal the reaction. UPC10, a mouse IgG2a myeloma, was used as a standard.

3.1.2. COS AND CHO CELLS TRANSFECTED WITH CHIMERIC OR CDR-GRAFTED OKT3 GENES
The assembly assay for chimeric or CDR-grafted antibody in COS cell supernatants was an ELISA with the following format:
96 well microtitre plates were coated with F(ab')2 goat anti-human IgG Fc. The plates were washed and samples added and incubated for 1 hour at room temperature. The plates were washed and monoclonal mouse anti-human kappa chain was added for 1 hour at room temperature.
The plates were washed and F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added. Enzyme substrate was added to reveal the reaction. Chimeric B72.3 (IgG4) (ref. 13) was used as a standard. The use of a monoclonal anti-kappa chain in this assay allows grafted antibodies to be read from the chimeric standard.

3.2. ASSAY FOR ANTIGEN BINDING ACTIVITY
Material from COS cell supernatants was assayed for OKT3 antigen binding activity onto CD3 positive cells in a direct assay. The procedure was as follows:
HUT 78 cells (human T cell line, CD3 positive) were maintained in culture. Monolayers of HUT 78 cells were prepared onto 96 well ELISA plates using poly-L-lysine and glutaraldehyde. Samples were added to the monolayers for 1 hour at room temperature.
The plates were washed gently using PBS. F(ab')2 goat anti-human IgG Fc (HRPO conjugated) or F-(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added as appropriate for humanised or mouse samples. Substrate was added to reveal the reaction.
The negative control for the cell-based assay was chimeric B72.3. The positive control was mouse Orthomune OKT3 or chimeric OKT3, when available. This cell-based assay was difficult to perform, and an alternative assay was developed for CDR-grafted OKT3 which was more sensitive and easier to carry out.
In this system CDR-grafted OKT3 produced by COS cells was tested for its ability to bind to the CD3-positive HPB-ALL (human peripheral blood acute lymphocytic leukemia) cell line. It was also tested for its ability to block the binding of murine OKT3 to these cells. Binding was measured by the following

procedure: HPB-ALL cells were harvested from tissue culture. Cells were incubated at 4°C for 1 hour with various dilutions of test antibody, positive control antibody, or negative control antibody. The cells were washed once and incubated at 4°C for 1 hour with an FITC-labelled goat anti-human IgG (Fc-specific, mouse absorbed). The cells were washed twice and analysed by cytofluorography. Chimeric OKT3 was used as a positive control for direct binding. Cells incubated with mock- transfected COS cell supernatant, followed by the FITC-labelled goat anti-human IgG, provided the negative control. To test the ability of CDR-grafted OKT3 to block murine OKT3 binding, the HPB-ALL cells were incubated at 4°C for 1 hour with various dilutions of test antibody or control antibody. A fixed saturating amount of FITC OKT3 was added. The samples were incubated for 1 hour at 4°C, washed twice and analysed by cytofluorography. FITC-labelled OKT3 was used as a positive control to determine maximum binding. Unlabelled murine OKT3 served as a reference standard for blocking. Negative controls were unstained cells with or without mock-transfected cell supernatant. The ability of the CDR-grafted OKT3 light chain to bind CD3-positive cells and block the binding of murine OKT3 was initially tested in combination with the chimeric OKT3 heavy chain. The chimeric OKT3 heavy chain is composed of the murine OKT3 variable region and the human IgG4 constant region. The chimeric heavy chain gene is expressed in the same expression vector used for the CDR-grafted genes. The CDR-grafted light chain expression vector and the chimeric heavy chain expression vector were co-transfected into COS cells. The fully chimeric OKT3 antibody (chimeric light chain and chimeric heavy chain) was found to be fully capable of binding to CD3 positive cells and blocking the binding of murine OKT3 to these cells.

3.3 DETERMINATION OF RELATIVE BINDING AFFINITY

The relative binding affinities of CDR-grafted anti-CD3 monoclonal antibodies were determined by competition binding (ref. 6) using the HPB-ALL human T cell line as a source of CD3 antigen, and fluorescein-conjugated murine OKT3 (Fl-OKT3) of known binding affinity as a tracer antibody. The binding affinity of Fl-OKT3 tracer antibody was determined by a direct binding assay in which increasing amounts of Fl-OKT3 were incubated with HPB-ALL (5×10^5) in PBS with 5% foetal calf serum for 60 min. at 4°C. Cells were washed, and the fluorescence intensity was determined on a FACScan flow cytometer calibrated with quantitative microbead standards (Flow Cytometry Standards, Research Triangle Park, NC). Fluorescence intensity per antibody molecule (F/P ratio) was determined by using microbeads which have a predetermined number of mouse IgG antibody binding sites (Simply Cellular beads, Flow Cytometry Standards). F/P equals the fluorescence intensity of beads saturated with Fl-OKT3 divided by the number of binding sites per bead. The amount of bound and free Fl-OKT3 was calculated from the mean fluorescence intensity per cell, and the ratio of bound/free was plotted against the number of moles of antibody bound. A linear fit was used to determine the affinity of binding (absolute value of the slope).

For competitive binding, increasing amounts of competitor antibody were added to a sub-saturating dose of Fl-OKT3 and incubated with 5×10^5 HPB-ALL in 200 ml of PBS with 5% foetal calf serum, for 60 min at 4°C. The fluorescence intensities of the cells were measured on a FACScan flow cytometer calibrated with quantitative microbead standards. The concentrations of bound and free Fl-OKT3 were calculated. The affinities of competing anti-bodies were calculated from the equation $[X]-[OKT3] = (1/Kx) - (1/Ka)$, where Ka is the affinity of murine OKT3, Kx is the affinity of competitor X, [ ] is the concentration of competitor antibody at which bound/free binding is R/2, and R is the maximal bound/free binding.

4. cDNA LIBRARY CONSTRUCTION

4.1. mRNA PREPARATION AND cDNA SYNTHESIS

OKT3 producing cells were grown as described above and 1.2×10^9 cells harvested and mRNA extracted using the guanidinium/LiCl extraction procedure. cDNA was prepared by priming from Oligo-dT to generate full length cDNA. The cDNA was methylated and EcoR1 linkers added for cloning.

4.2. LIBRARY CONSTRUCTION

The cDNA library was ligated to pSP65 vector DNA which had been EcoR1 cut and the 5' phosphate groups removed by calf intestinal phosphatase (EcoR1/CIP). The ligation was used to transform high transformation efficiency Escherichia coli (E.coli) HB101. A cDNA library was prepared. 3600 colonies were screened for the light chain and 10000 colonies were screened for the heavy chain.

5. SCREENING

E.coli colonies positive for either heavy or light chain probes were identified by oligonucleotide screening using the oligonucleotides: 5' TCCAGATGTTAACTGCTCAC for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5' CAGGGGCCAGTGGATGGATAGAC for the heavy chain which is complementary to a sequence in the mouse IgG2a constant CH1 domain region. 12

light chain and 9 heavy chain clones were identified and taken for second round screening. Positive clones from the second round of screening were grown up and DNA prepared. The sizes of the gene inserts were estimated by gel electrophoresis and inserts of a size capable of containing a full length cDNA were subcloned into M13 for DNA sequencing.

6. DNA SEQUENCING

Clones representing four size classes for both heavy and light chains were obtained in M13. DNA sequence for the 5' untranslated regions, signal sequences, variable regions and 3' untranslated regions of full length cDNAs [Figures 1(a) and 2(a)] were obtained and the corresponding amino acid sequences predicted [(Figures 1(b) and 2(b)]. In Figure 1(a) the untranslated DNA regions are shown in uppercase, and in both Figures 1 and 2 the signal sequences are underlined.

7. CONSTRUCTION OF cDNA EXPRESSION VECTORS

Celltech expression vectors are based on the plasmid pEE6hCMV (ref. 14). A polylinker for the insertion of genes to be expressed has been introduced after the major immediate early promoter/enhancer of the human Cytomegalovirus (hCMV). Marker genes for selection of the plasmid in transfected eukaryotic cells can be inserted as BamH1 cassettes in the unique BamH1 site of pEE6 hCMV; for instance, the neo marker to provide pEE6 hCMV neo. It is usual practice to insert the neo and gpt markers prior to insertion of the gene of interest, whereas the GS marker is inserted last because of the presence of internal EcoR1 sites in the cassette.

The selectable markers are expressed from the SV40 late promoter which also provides an origin of replication so that the vectors can be used for expression in the COS cell transient expression system.

The mouse sequences were excised from the M13 based vectors described above as EcoR1 fragments and cloned into either pEE6-hCMV-neo for the heavy chain and into EE6-hCMV-gpt for the light chain to yield vectors pJA136 and pJA135 respectively.

8. EXPRESSION OF cDNAS IN COS CELLS

Plasmids pJA135 and pJA136 were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to T-cell enriched lymphocytes. Metabolic labelling experiments using ^{35}S methionine showed expression and assembly of heavy and light chains.

9. CONSTRUCTION OF CHIMERIC GENES

Construction of chimeric genes followed a previously described strategy [Whittle et al (ref. 13)]. A restriction site near the 3' end of the variable domain sequence is identified and used to attach an oligonucleotide adapter coding for the remainder of the mouse variable region and a suitable restriction site for attachment to the constant region of choice.

9.1. LIGHT CHAIN GENE CONSTRUCTION

The mouse light chain cDNA sequence contains an Aval site near the 3' end of the variable region [Fig. 1(a)]. The majority of the sequence of the variable region was isolated as a 396 bp. EcoR1-Aval fragment. An oligonucleotide adapter was designed to replace the remainder of the 3' region of the variable region from the Aval site and to include the 5' residues of the human constant region up to and including a unique Nar1 site which had been previously engineered into the constant region.

A Hind111 site was introduced to act as a marker for insertion of the linker.

The linker was ligated to the V_L fragment and the 413 bp EcoR1-Nar1 adapted fragment was purified from the ligation mixture.

The constant region was isolated as an Nar1-BamH1 fragment from an M13 clone NW361 and was ligated with the variable region DNA into an EcoR1/BamH1/C1P pSP65 treated vector in a three way reaction to yield plasmid JA143. Clones were isolated after transformation into E.coli and the linker and junction sequences were confirmed by the presence of the Hind111 site and by DNA sequencing.

9.2 LIGHT CHAIN GENE CONSTRUCTION - VERSION 2

The construction of the first chimeric light chain gene produces a fusion of mouse and human amino acid sequences at the variable-constant region junction. In the case of the OKT3 light chain the amino acids at the chimera junction are:

........Leu-Glu-Ile-Asn-Arg/ -/Thr-Val-Ala -Ala
VARIABLE CONSTANT

This arrangement of sequence introduces a potential site for Asparagine (Asn) linked (N-linked) glycosylation at the V-C junction. Therefore, a second version of the chimeric light chain oligonucleotide adapter was designed in which the threonine (Thr), the first amino acid of the human constant region, was replaced with the equivalent amino acid from the mouse constant region, Alanine (Ala).

An internal Hind111 site was not included in this adapter, to differentiate the two chimeric light chain genes.

The variable region fragment was isolated as a 376 bp EcoR1-Aval fragment. The oligonucleotide linker was ligated to Nar1 cut pNW361 and then the adapted 396bp constant region was isolated after recutting the modified pNW361 with EcoR1. The variable region fragment and the modified constant region fragment were ligated directly into EcoR1/C1P treated pEE6hCMVneo to yield pJA137. Initially all clones examined had the insert in the incorrect orientation. Therefore, the insert was re-isolated and recloned to turn the insert round and yield plasmid pJA141. Several clones with the insert in the correct orientation were obtained and the adapter sequence of one was confirmed by DNA sequencing

9.3. HEAVY CHAIN GENE CONSTRUCTION

9.3.1. CHOICE OF HEAVY CHAIN GENE ISOTYPE

The constant region isotype chosen for the heavy chain was human IgG4.

9.3.2. GENE CONSTRUCTION

The heavy chain cDNA sequence showed a Ban1 site near the 3' end of the variable region [Fig. 2(a)-]. The majority of the sequence of the variable region was isolated as a 426bp. EcoR1/C1P/Ban1 fragment. An oligonucleotide adapter was designated to replace the remainder of the 3' region of the variable region from the Ban1 site up to and including a unique HindIII site which had been previously engineered into the first two amino acids of the constant region.

The linker was ligated to the V_H fragment and the EcoR1-Hind111 adapted fragment was purified from the ligation mixture. The variable region was ligated to the constant region by cutting pJA91 with EcoR1 and Hind111 removing the intron fragment and replacing it with the V_H to yield pJA142. Clones were isolated after transformation into E.coli JM101 and the linker and junction sequences were confirmed by DNA sequencing. (N.B. The Hind111 site is lost on cloning).

10. <u>CONSTRUCTION OF CHIMERIC EXPRESSION VECTORS</u>

10.1. neo AND gpt VECTORS

The chimeric light chain (version 1) was removed from pJA143 as an EcoR1 fragment and cloned into EcoR1/C1P treated pEE6hCMVneo expression vector to yield pJA145. Clones with the insert in the correct orientation were identified by restriction mapping.

The chimeric light chain (version 2) was constructed as described above.

The chimeric heavy chain gene was isolated from pJA142 as a 2.5Kbp EcoR1/BamH1 fragment and cloned into the EcoR1/Bcl1/C1P treated vector fragment of a derivative of pEE6hCMVgpt to yield plasmid pJA144.

10.2. GS SEPARATE VECTORS

GS versions of pJA141 and pJA144 were constructed by replacing the neo and gpt cassettes by a BamH1/Sa11/C1P treatment of the plasmids, isolation of the vector fragment and ligation to a GS-containing fragment from the plasmid pRO49 to yield the light chain vector pJA179 and the heavy chain vector pJA180.

10.3. GS SINGLE VECTOR CONSTRUCTION

Single vector constructions containing the cL (chimeric light), cH (chimeric heavy) and GS genes on one plasmid in the order cL-cH-GS, or cH-cL-GS and with transcription of the genes being head to tail e.g. cL>cH>GS were constructed. These plasmids were made by treating pJA179 or pJA180 with BamH1/C1P and ligating in a Bgl11/Hind111 hCMV promoter cassette along with either the Hind111/BamH1 fragment from pJA141 into pJA180 to give the cH-cL-GS plasmid pJA182 or the Hind111/BamH1 fragment from pJA144 into pJA179 to give the cL-cH-GS plasmid pJA181.

11. <u>EXPRESSION OF CHIMERIC GENES</u>

<u>11.1. EXPRESSION IN COS CELLS</u>

The chimeric antibody plasmid pJA145 (cL) and pJA144 (cH) were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to the HUT 78 human T-cell line. Metabolic labelling experiments using ^{35}S methionine showed expression and assembly of heavy and light chains. However the light chain mobility seen on reduced gels suggested that the potential glycosylation site was being glycosylated. Expression in COS cells in the presence of tunicamycin showed a reduction in size of the light chain to that shown for control chimeric antibodies and the OKT3 mouse light chain. Therefore JA141 was constructed and expressed. In this case the light chain did not show an aberrant mobility or a size shift in the presence or absence of tunicamycin. This second version of the chimeric light chain, when expressed in association with chimeric heavy (cH) chain, produced antibody which showed good binding to HUT

78 cells. In both cases antigen binding was equivalent to that of the mouse antibody.

11.2 EXPRESSION IN CHINESE HAMSTER OVARY (CHO) CELLS Stable cell lines have been prepared from plasmids PJA141/pJA144 and from pJA179/pJA180, pJA181 and pJA182 by transfection into CHO cells.

12. CDR-GRAFTING

The approach taken was to try to introduce sufficient mouse residues into a human variable region framework to generate antigen binding activity comparable to the mouse and chimeric antibodies.

12.1. VARIABLE REGION ANALYSIS

From an examination of a small database of structures of antibodies and antigen-antibody complexes it is clear that only a small number of antibody residues make direct contact with antigen. Other residues may contribute to antigen binding by positioning the contact residues in favourable configurations and also by inducing a stable packing of the individual variable domains and stable interaction of the light and heavy chain variable domains.

The residues chosen for transfer can be identified in a number of ways:

(a) By examination of antibody X-ray crystal structures the antigen binding surface can be predominantly located on a series of loops, three per domain, which extend from the B-barrel framework.

(b) By analysis of antibody variable domain sequences regions of hypervariability [termed the Complementarity Determining Regions (CDRs) by Wu and Kabat (ref. 5)] can be identified. In the most but not all cases these CDRs correspond to, but extend a short way beyond, the loop regions noted above.

(c) Residues not identified by (a) and (b) may contribute to antigen binding directly or indirectly by affecting antigen binding site topology, or by inducing a stable packing of the individual variable domains and stabilising the inter-variable domain interaction. These residues may be identified either by superimposing the sequences for a given antibody on a known structure and looking at key residues for their contribution, or by sequence alignment analysis and noting "idiosyncratic" residues followed by examination of their structural location and likely effects.

12.1.1. LIGHT CHAIN

Figure 3 shows an alignment of sequences for the human framework region RE1 and the OKT3 light variable region. The structural loops (LOOP) and CDRs (KABAT) believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 13.1(c). Above the sequence in Figure 3 the residue type indicates the spatial location of each residue side chain, derived by examination of resolved structures from X-ray crystallography analysis. The key to this residue type designation is as follows:

N - near to CDR (From X-ray Structures)
P - Packing B - Buried Non-Packing
S - Surface E - Exposed
I - Interface * - Interface
- Packing/Part Exposed
? - Non-CDR Residues which may require to be left as Mouse sequence. Residues underlined in Figure 3 are amino acids. RE1 was chosen as the human framework because the light chain is a kappa chain and the kappa variable regions show higher homology with the mouse sequences than a lambda light variable region, e.g. KOL (see below). RE1 was chosen in preference to another kappa light chain because the X-ray structure of the light chain has been determined so that a structural examination of individual residues could be made.

12.1.2. HEAVY CHAIN

Similarly Figure 4 shows an alignment of sequences for the human framework region KOL and the OKT3 heavy variable region. The structural loops and CDRs believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 12.1(c). The residue type key and other indicators used in Figure 4 are the same as those used in Figure 3. KOL was chosen as the heavy chain framework because the X-ray structure has been determined to a better resolution than, for example, NEWM and also the sequence alignment of OKT3 heavy variable region showed a slightly better homology to KOL than to NEWM.

12.2. DESIGN OF VARIABLE GENES

The variable region domains were designed with mouse variable region optimal codon usage [Grantham and Perrin (ref. 15)] and used the B72.3 signal sequences [Whittle et al (ref. 13)]. The sequences were designed to be attached to the constant region in the same way as for the chimeric genes described above. Some constructs contained the "Kozak consensus sequence" [Kozak (ref. 16)] directly linked to the 5' of the signal sequence in the gene. This sequence motif is believed to have a beneficial role in translation initiation in eukaryotes.

12.3. GENE CONSTRUCTION

To build the variable regions, various strategies are available. The sequence may be assembled by using oligonucleotides in a manner similar to Jones et al (ref. 17) or by simultaneously replacing all of the CDRs or loop regions by oligonucleotide directed site specific mutagenesis in a manner similar to Verhoeyen et al (ref. 2). Both strategies were used and a list of constructions is set out in Tables 1 and 2 and Figures 4 and 5. It was noted in several cases that the mutagenesis approach led to deletions and rearrangements in the gene being remodelled, while the success of the assembly approach was very sensitive to the quality of the oligonucleotides.

13. CONSTRUCTION OF EXPRESSION VECTORS

Genes were isolated from M13 or SP65 based intermediate vectors and cloned into pEE6hCMVneo for the light chains and pEE6hCMVgpt for the heavy chains in a manner similar to that for the chimeric genes as described above.

TABLE 1 CDR-GRAFTED GENE CONSTRUCTS

CODE	MOUSE SEQUENCE CONTENT	METHOD OF CONSTRUCTION	KOZAK SEQUENCE	
			-	+
LIGHT CHAIN	ALL HUMAN FRAMEWORK RE1			
121	26-32, 50-56, 91-96 inclusive	SDM and gene assembly	+	n.d.
121A	26-32, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	n.d.	+
121B	26-32, 50-56, 91-96 inclusive + 46, 47	Partial gene assembly	n.d.	+
221	24-24, 50-56, 91-96 inclusive	Partial gene assembly	+	+
221A	24-34, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	+	+
221B	24-34, 50-56, 91-96 inclusive +1, 3	Partial gene assembly	+	+
221C	24-34, 50-56, 91-96 inclusive	Partial gene assembly	+	+
HEAVY CHAIN	ALL HUMAN FRAMEWORK KOL			
121	26-32, 50-56, 95-100B inclusive	Gene assembly	n.d.	+
131	26-32, 50-58, 95-100B inclusive	Gene assembly	n.d.	+
141	26-32, 50-65, 95-100B inclusive	Partial gene assembly	+	n.d.
321	26-35, 50-56, 95-100B inclusive	Partial gene assembly	+	n.d.
331	26-35, 50-58, 95-100B inclusive	Partial gene assembly	+	
		Gene assembly		+
341	26-35, 50-65, 95-100B inclusive	SDM	+	
		Partial gene assembly		+
341A	26-35, 50-65, 95-100B inclusive +6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91 (+63 = human)	Gene assembly	n.d.	+
341B	26-35, 50-65, 95-100B inclusive + 48, 49, 71, 73, 76, 78, 88, 91 (+63 + human)	Gene assembly	n.d.	+

KEY

n.d.	not done
SDM	Site directed mutagenesis
Gene assembly	Variable region assembled entirely from oligonucleotides
Partial gene assembly	Variable region assembled by combination of restriction fragments either from other genes originally created by SDM and gene assembly or by oligonucleotide assembly of part of the variable region and reconstruction with restriction fragments from other genes originally created by SDM and gene assembly

14. EXPRESSION OF CDR-GRAFTED GENES

14.1. PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED LIGHT (gL) CHAINS WITH MOUSE HEAVY (mH) OR CHIMERIC HEAVY (cH) CHAINS

All gL chains, in association with mH or cH produced reasonable amounts of antibody. Insertion of the Kozak consensus sequence at a position 5' to the ATG (kgL constructs) however, led to a 2-5 fold improvement in net expression. Over an extended series of experiments expression levels were raised from approximately 200ng/ml to approximately 500 ng/ml for kgL/cH or kgL/mH combinations.

When direct binding to antigen on HUT 78 cells was measured, a construct designed to include mouse sequence based on loop length (gL121) did not lead to active antibody in association with mH or cH. A construct designed to include mouse sequence based on Kabat CDRs (gL221) demonstrated some weak binding in association with mH or cH. However, when framework residues 1, 3, 46, 47 were changed from the human to the murine OKT3 equivalents based on the arguments outlined in Section 12.1 antigen binding was demonstrated when both of the new constructs, which were termed 121A and 221A were co-expressed with cH. When the effects of these residues were examined in more detail, it appears that residues 1 and 3 are not major contributing residues as the product of the gL221B gene shows little detectable binding activity in association with cH. The light chain product of gL221C, in which mouse sequences are present at 46 and 47, shows good binding activity in association with cH.

14.2 PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED HEAVY (gH) CHAINS WITH MOUSE LIGHT (mL) OR CHIMERIC LIGHT (cL) CHAINS

Expression of the gH genes proved to be more difficult to achieve than for gL. First, inclusion of the Kozak sequence appeared to have no marked effect on expression of gH genes. Expression appears to be slightly improved but not to the same degree as seen for the grafted light chain.

Also, it proved difficult to demonstrate production of expected quantities of material when the loop choice (amino acid 26-32) for CDR1 is used, e.g. gH121, 131, 141 and no conclusions can be drawn about these constructs.

Moreover, co-expression of the gH341 gene with cL or mL has been variable and has tended to produce lower amounts of antibody than the cH/cL or mH/mL combinations. The alterations to gH341 to produce gH341A and gH341B lead to improved levels of expression.

This may be due either to a general increase in the fraction of mouse sequence in the variable region, or to the alteration at position 63 where the residue is returned to the human amino acid Valine (Val) from Phenylalanine (Phe) to avoid possible internal packing problems with the rest of the human framework. This arrangement also occurs in gH331 and gH321.

When gH321 or gH331 were expressed in association with cL, antibody was produced but antibody binding activity was not detected.

When the more conservative gH341 gene was used antigen binding could be detected in association with cL or mL, but the activity was only marginally above the background level. When further mouse residues were substituted based on the arguments in 12.1, antigen binding could be clearly demonstrated for the antibody produced when kgH341A and kgH341B were expressed in association with cL.

14.3 PRODUCTION OF FULLY CDR-GRAFTED ANTIBODY

The kgL221A gene was co-expressed with kgH341, kgH341A or kgH341B. For the combination kgH221A/kgH341 very little material was produced in a normal COS cell expression.

For the combinations kgL221A/kgH341A or kgH221A/kgH341B amounts of antibody similar to gL/cH was produced.

In several experiments no antigen binding activity could be detected with kgH221A/gH341 or kgH221A/kgH341 combinations, although expression levels were very low.

Antigen binding was detected when kgL221A/kgH341A or kgH221A/kgH341B combinations were expressed. In the case of the antibody produced from the kgL221A/kgH341A combination the antigen binding was very similar to that of the chimeric antibody.

An analysis of the above results is given below.

15. DISCUSSION OF CDR-GRAFTING RESULTS

In the design of the fully humanised antibody the aim was to transfer the minimum number of mouse amino acids that would confer antigen binding onto a human antibody framework.

15.1. LIGHT CHAIN

15.1.1. EXTENT OF THE CDRs

For the light chain the regions defining the loops known from structural studies of other antibodies to contain the antigen contacting residues, and those hypervariable sequences defined by Kabat et al (refs. 4 and 5) as Complementarity Determining Regions (CDRs) are equivalent for CDR2. For CDR1 the hypervariable region extends from residues 24-34 inclusive while the structural loop extends from 26-32 inclusive. In the case of OKT3 there is only one amino acid difference between the two options, at amino acid 24, where the mouse sequence is a serine and the human framework RE1 has glutamine. For CDR3 the loop extends from residues 91-96 inclusive while the

Kabat hypervariability extends from residues 89-97 inclusive. For OKT3 amino acids 89, 90 and 97 are the same between OKT3 and RE1 (Fig. 3). When constructs based on the loop choice for CDR1 (gL121) and the Kabat choice (gL221) were made and co-expressed with mH or cH no evidence for antigen binding activity could be found for gL121, but trace activity could be detected for the gL221, suggesting that a single extra mouse residue in the grafted variable region could have some detectable effect. Both gene constructs were reasonably well expressed in the transient expression system.

15.1.2. FRAMEWORK RESIDUES

The remaining framework residues were then further examined, in particular amino acids known from X-ray analysis of other antibodies to be close to the CDRs and also those amino acids which in OKT3 showed differences from the consensus framework for the mouse subgroup (subgroup VI) to which OKT3 shows most homology. Four positions 1, 3, 46 and 47 were identified and their possible contribution was examined by substituting the mouse amino acid for the human amino acid at each position. Therefore gL221A (gL221 + D1Q, Q3V, L46R, L47W, see Figure 3 and Table 1) was made, cloned in EE6hCMVneo and co-expressed with cH (pJA144). The resultant antibody was well expressed and showed good binding activity. When the related genes gL221B (gL221 + D1Q, Q3V) and gL221C (gL221 + L46R, L47W) were made and similarly tested, while both genes produced antibody when co-expressed with cH, only the gL221C/cH combination showed good antigen binding. When the gL121A (gL121 + D1Q, Q3V, L46R, L47W) gene was made and co-expressed with cH, antibody was produced which also bound to antigen.

15.2. HEAVY CHAIN

15.2.1. EXTENT OF THE CDRs

For the heavy chain the loop and hypervariability analyses agree only in CDR3. For CDR1 the loop region extends from residues 26-32 inclusive whereas the Kabat CDR extends from residues 31-35 inclusive. For CDR2 the loop region is from 50-58 inclusive while the hypervariable region covers amino acids 50-65 inclusive. Therefore humanised heavy chains were constructed using the framework from antibody KOL and with various combinations of these CDR choices, including a shorter choice for CDR2 of 50-56 inclusive as there was some uncertainty as to the definition of the end point for the CDR2 loop around residues 56 to 58. The genes were co-expressed with mL or cL initially. In the case of the gH genes with loop choices for CDR1 e.g. gH121, gH131, gH141 very little antibody was produced in the culture supernatants. As no free light chain was detected it was presumed that the antibody was being made and assembled inside the cell but that the heavy chain was aberrant in some way, possibly incorrectly folded, and therefore the antibody was being degraded internally. In some experiments trace amounts of antibody could be detected in ^{35}S labelling studies.

As no net antibody was produced, analysis of these constructs was not pursued further.

When, however, a combination of the loop choice and the Kabat choice for CDR1 was tested (mouse amino acids 26-35 inclusive) and in which residues 31 (Ser to Arg), 33 (Ala to Thr), and 35 (Tyr to His) were changed from the human residues to the mouse residue and compared to the first series, antibody was produced for gH321, kgH331 and kgH341 when co-expressed with cL. Expression was generally low and could not be markedly improved by the insertion of the Kozak consensus sequence 5' to the ATG of the signal sequence of the gene, as distinct from the case of the gL genes where such insertion led to a 2-5 fold increase in net antibody production. However, only in the case of gH341/mL or kgH341/cL could marginal antigen binding activity be demonstrated. When the kgH341 gene was co-expressed with kgL221A, the net yield of antibody was too low to give a signal above the background level in the antigen binding assay.

15.2.2. FRAMEWORK RESIDUES

As in the case of the light chain the heavy chain frameworks were re-examined. Possibly because of the lower initial homology between the mouse and human heavy variable domains compared to the light chains, more amino acid positions proved to be of interest. Two genes kgH341A and kgH341B were constructed, with 11 or 8 human residues respectively substituted by mouse residues compared to gH341, and with the CDR2 residue 63 returned to the human amino acid potentially to improve domain packing. Both showed antigen binding when combined with cL or kgL221A, the kgH341A gene with all 11 changes appearing to be the superior choice.

15.3 INTERIM CONCLUSIONS

It has been demonstrated, therefore, for OKT3 that to transfer antigen binding ability to the humanised antibody, mouse residues outside the CDR regions defined by the Kabat hypervariability or structural loop choices are required for both the light and heavy chains. Fewer extra residues are needed for the light chain, possibly due to the higher initial homology between the mouse and human kappa variable regions.

Of the changes seven (1 and 3 from the light chain and 6, 23, 71, 73 and 76 from the heavy chain) are predicted from a knowledge of other antibody structures to be either partly exposed or on the antibody surface. It has been shown here that residues 1 and 3 in the light chain are not absolutely required to be the mouse sequence; and for the heavy chain the gH341B heavy chain in combination with the 221A light chain generated only weak binding activity. Therefore the presence of the 6, 23 and 24 changes are important to maintain a binding affinity similar to that of the murine antibody. It was important, therefore, to further study the individual contribution of othe other 8 mouse residues of the kgH341A gene compared to kgH341.

16. FURTHER CDR-GRAFTING EXPERIMENTS

Additional CDR-grafted heavy chain genes were prepared substantially as described above. With reference to Table 2 the further heavy chain genes were based upon the gh341 (plasmid pJA178) and gH341A (plasmid pJA185) with either mouse OKT3 or human KOL residues at 6, 23, 24, 48, 49, 63, 71, 73, 76, 78, 88 and 91, as indicated. The CDR-grafted light chain genes used in these further experiments were gL221, gL221A, gL221B and gL221C as described above.

TABLE 2

OKT3 HEAVY CHAIN CDR GRAFTS

1. gH341 and derivatives

RES NUM	6	23	24	48	49	63	71	73	76	78	88	91	
OKT3vh	Q	K	A	I	G	F	T	K	S	A	A	Y	
gH341	E	S	S	V	A	F	R	N	N	L	G	F	JA178
gH341A	Q	K	A	I	G	V	T	K	S	A	A	Y	JA185
gH341E	Q	K	A	I	G	V	T	K	S	A	G	G	JA198
gH341*	Q	K	A	I	G	V	T	K	N	A	G	F	JA207
gH341*	Q	K	A	I	G	V	R	N	N	A	G	F	JA209
gH341D	Q	K	A	I	G	V	T	K	N	L	G	F	JA197
gH341*	Q	K	A	I	G	V	R	N	N	L	G	F	JA199
gH341C	Q	K	A	V	A	F	R	N	N	L	G	F	JA184
gH341*	Q	S	A	I	G	V	T	K	S	A	A	Y	JA203
gH341*	E	S	A	I	G	V	T	K	S	A	A	Y	JA205
gH341B	E	S	S	I	G	V	T	K	S	A	A	Y	JA183
gH341*	Q	S	A	I	G	V	T	K	S	A	G	F	JA204
gH341*	E	S	A	I	G	V	T	K	S	A	G	F	JA206
gH341*	Q	S	A	I	G	V	T	K	N	A	G	F	JA208
KOL	E	S	S	V	A		R	N	N	L	G	F	

OKT3 LIGHT CHAIN CDR GRAFTS

2. gL221 and derivatives

RES NUM	1	3	46	47	
OKT3vl	Q	V	R	W	
GL221	D	Q	L	L	DA221
gL221A	Q	V	R	W	DA221A
gL221B	Q	V	L	L	DA221B
GL221C	D	Q	R	W	DA221C
RE1	D	Q	L	L	

MURINE RESIDUES ARE UNDERLINED

The CDR-grafted heavy and light chain genes were co-expressed in COS cells either with one another in various combinations but also with the corresponding murine and chimeric heavy and light chain genes substantially as described above. The resultant antibody products were then assayed in binding and blocking assays with HPB-ALL cells as described above.

The results of the assays for various grafted heavy chains co-expressed with the gL221C light chain are given in Figures 7 and 8 (for the JA184, JA185, JA197 and JA198 constructs - see Table 2), in Figure 9 (for the JA183, JA184, JA185 and JA197 constructs) in Figure 10 (for the chimeric, JA185, JA199, JA204, JA205, JA207, JA208 and JA209 constructs) and in Figure 11 (for the JA183, JA184, JA185, JA198, JA203, JA205 and JA206 constructs).

The basic grafted product without any human to murine changes in the variable frameworks, i.e. gL221 co-expressed with gh341 (JA178), and also the "fully grafted" product, having most human to murine changes in the grafted heavy chain framework, i.e. gL221C co-expressed with gh341A (JA185), were assayed for relative binding affinity in a competition assay against murine OKT3 reference standard, using HPB-ALL cells. The assay used was as described above in section 3.3. The results obtained are given in Figure 12 for the basic grafted product and in Figure 13 for the fully grafted product. These results indicate that the basic grafted product has neglibible binding ability as compared with the OKT3 murine reference standard; whereas the "fully grafted" product has a binding ability very similar to that of the OKT3 murine reference standard.

The binding and blocking assay results indicate the following:
The JA198 and JA207 constructs appear to have the best binding characteristics and similar binding abilities, both substantially the same as the chimeric and fully grafted gH341A products. This indicates that positions 88 and 91 and position 76 are not highly critical for maintaining the OKT3 binding ability; whereas at least some of positions 6, 23, 24, 48, 49, 71, 73 and 78 are more important.

This is borne out by the finding that the JA209 and JA199, although of similar binding ability to one another, are of lower binding ability than the JA198 and JA207 constructs. This indicates the importance of having mouse residues at positions 71, 73 and 78, which are either completely or partially human in the JA199 and JA209 constructs respectively.

Moreover, on comparing the results obtained for the JA205 and JA183 constructs it is seen that there is a decrease in binding going from the JA205 to the JA183 constructs. This indicates the importance of retaining a mouse residue at position 23, the only position changed between JA205 and JA183.

These and other results lead us to the conclusion that of the 11 mouse framework residues used in the gH341A (JA185) construct, it is important to retain mouse residues at all of positions 6, 23, 24, 48 and 49, and possibly for maximum binding affinity at 71, 73 and 78.

Similar Experiments were carried out to CDR-graft a number of the rodent antibodies including antibodies having specificity for CD4 (OKT4), ICAM-1 (R6-5), TAG72 (B72.3), and TNFα(61E71, 101.4, hTNF1, hTNF2 and hTNF3).

EXAMPLE 2

CDR-GRAFTING OF A MURINE ANTI-CD4 T CELL RECEPTOR ANTIBODY, OKT4A

Anti OKT4A CDR-grafted heavy and light chain genes were prepared, expressed and tested substantially as described above in Example 1 for CDR-grafted OKT3. The CDR grafting of OKT4A is described in detail in Ortho patent application PCT/GB 90......... of even date herewith entitled "Humanised Antibodies". The disclosure of this Ortho patent application PCT/GB 90 .... is incorporated herein by reference. A number of CDR-grafted OKT4 antibodies have been prepared. Presently the CDR-grafted OKT4A of choice is the combination of the grafted light chain LCDR2 and the grafted heavy chain HCDR10.

THE LIGHT CHAIN

The human acceptor framework used for the grafted light chains was RE1. The preferred LCDR2 light chain has human to mouse changes at positions 33, 34, 38, 49 and 89 in addition to the structural loop CDRs. Of these changed positions, positions 33, 34 and 89 fall within the preferred extended CDRs of the present invention (positions 33 and 34 in CDR1 and position 89 in CDR3).
The human to murine changes at positions 38 and 49 corresponds to positions at which the amino acid residues are preferably donor murine amino acid residues in accordance with the present invention.
A comparison of the amino acid sequences of the donor murine light chain variable domain and the RE1 human acceptor light chain variable further reveals that the murine and human residues are identical at all of positions 46, 48 and 71 and at all of positions 2, 4, 6, 35, 36, 44, 47, 62, 64-69, 85, 87, 98, 99 and 101 and 102. However the amino acid residue at position 58 in LCDR2 is the human RE1 framework residue not the mouse OKT4 residue as would be preferred in accordance with the present invention.

THE HEAVY CHAIN

The human acceptor framework used for the grafted heavy chains was KOL.
The preferred CDR graft HCDR10 heavy chain has human to mouse changes at positions 24, 35, 57, 58, 60, 88 and 91 in addition to the structural loop CDRs.
Of these positions, positions 35 (CDR1) and positions 57, 58 and 60 (CDR2) fall within the preferred extended CDRs of the present invention. Also the human to mouse change at position 24 corresponds to a position at which the amino acid residue is a donor murine residue in accordance with the present invention. Moreover, the human to mouse changes at positions 88 and 91 correspond to positions at which the amino acid residues are optionally donor murine residues.
Moreover, a comparison of the murine OKT4A and human KOL heavy chain variable amino acid sequences reveals that the murine and human residues are identical at all of positions 23, 49, 71, 73 and 78 and at all of positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.
Thus the OKT4A CDR-grafted heavy chain HCDR10 corresponds to a particularly preferred embodiment according to the present invention.

EXAMPLE 3

CDR-GRAFTING OF AN ANTI-MUCIN SPECIFIC MURINE ANTIBODY, B72.3

The cloning of the genes coding for the anti-mucin specific murine monoclonal antibody B72.3 and the preparation of B72.3 mouse-human chimeric antibodies has been described previously (ref. 13 and WO 89/01783). CDR-grafted versions of B72.3 were prepared as follows.

(a) B72.3 Light Chain

CDR-grafting of this light chain was accomplished by direct transfer of the murine CDRs into the framework of the human light chain RE1. The regions transferred were:

CDR Number	Residues
1	24-34
2	50-56
3	90-96

The activity of the resulting grafted light chain was assessed by co-expression in COS cells, of genes for the combinations:
B72.3 cH/B72.3 cL
and B72.3 cH/B72.3 gL
Supernatants were assayed for antibody concentration and for the ability to bind to microtitre plates coated with mucin. The results obtained indicated that, in combination with the B72.3 cH chain, B72.3 cL and B72.3 gL had similar binding properties.

Comparison of the murine B72.3 and REI light chain amino acid sequences reveals that the residues are identical at positions 46, 58 and 71 but are different at position 48. Thus changing the human residue to the donor mouse residue at position 48 may further improve the binding characteristics of the CDR-grafted Light chain, (B72.3 gL) in accordance with the present invention.

(b) B72.3 heavy chain

i. Choice of framework

At the outset it was necessary to make a choice of human framework. Simply put, the question was as follows: Was it necessary to use the framework regions from an antibody whose crystal structure was known or could the choice be made on some other criteria?
For B72.3 heavy chain, it was reasoned that, while knowledge of structure was important, transfer of the CDRs from mouse to human frameworks might be facilitated if the overall homology between the donor and receptor frameworks was maximised. Comparison of the B72.3 heavy chain sequence with those in Kabat (ref. 4) for human heavy chains showed clearly that B72.3 had poor homology for KOL and NEWM (for which crystal structures are available) but was very homologous to the heavy chain for EU.
On this basis, EU was chosen for the CDR-grafting and the following residues transferred as CDRs.

CDR Number	Residues
1	27-36
2	50-63
3	93-102

Also it was noticed that the FR4 region of EU was unlike that of any other human (or mouse) antibody. Consequently, in the grafted heavy chain genes this was also changed to produce a "consensus" human sequence. (Preliminary experiments showed that grafted heavy chain genes containing the EU FR4 sequence expressed very poorly in transient expression systems.)

ii. Results with grafted heavy chain genes

Expression of grafted heavy chain genes containing all human framework regions with either gL or cL genes produced a grafted antibody with little ability to bind to mucin. The grafted antibody had about 1% the activity of the chimeric antibody. In these experiments, however, it was noted that the activity of the grafted antibody could be increased to ~ 10% of B72.3 by exposure to pHs of 2-3.5.

This observation provided a clue as to how the activity of the grafted antibody could be improved without acid treatment. It was postulated that acid exposure brought about the protonation of an acidic residue (pKa of aspartic acid = 3.86 and of glutamine acid = 4.25) which in turn caused a change in structure of the CDR loops, or allowed better access of antigen.

From comparison of the sequences of B72.3 (ref. 13) and EU (refs. 4 and 5), it was clear that, in going from the mouse to human frameworks, only two positions had been changed in such a way that acidic residues had been introduced. These positions are at residues 73 and 81, where K to E and Q to E changes had been made, respectively.

Which of these positions might be important was determined by examining the crystal structure of the KOL antibody. In KOL heavy chain, position 81 is far removed from either of the CDR loops.

Position 73, however, is close to both CDRs 1 and 3 of the heavy chain and, in this position it was possible to envisage that a K to E change in this region could have a detrimental effect on antigen binding.

iii. Framework changes in B72.3 gH gene

On the basis of the above analysis, E73 was mutated to a lysine (K). It was found that this change had a dramatic effect on the ability of the grafted Ab to bind to mucin. Further the ability of the grafted B72.3 produced by the mutated gH/gL combination to bind to mucin was similar to that of the B72.3 chimeric antibody.

iv. Other framework changes

In the course of the above experiments, other changes were made in the heavy chain framework regions. Within the accuracy of the assays used, none of the changes, either alone or together, appeared beneficial.

v. Other

All assays used measured the ability of the grafted Ab to bind to mucin and, as a whole, indicated that the single framework change at position 73 is sufficient to generate an antibody with similar binding properties to B72.3.

Comparison of the B72.3 murine and EU heavy chain sequences reveals that the mouse and human residues are identical at positions 23, 24, 71 and 78.

Thus the mutated CDR-grafted B72.3 heavy chain corresponds to a preferred embodiment of the present invention.

EXAMPLE 4

CDR-GRAFTING OF A MURINE ANTI-ICAM-1 MONOCLONAL ANTIBODY

A murine antibody, R6-5-D6 (EP 0314863) having specificity for Intercellular Adhesion Molecule 1 (ICAM-1) was CDR-grafted substantially as described above in previous examples. This work is described in greater detail in co-pending application, British Patent Application No. 9009549.8, the disclosure of which is incorporated herein by reference.

The human EU framework was used as the acceptor framework for both heavy and light chains. The CDR-grafted antibody currently of choice is provided by co-expression of grafted light chain gL221A and grafted heavy chain gH341D which has a binding affinity for ICAM 1 of about 75% of that of the corresponding mouse-human chimeric antibody.

LIGHT CHAIN

gL221A has murine CDRs at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition several framework residues are also the murine amino acid. These residues were chosen after consideration of the possible contribution of these residues to domain packing and stability of the conformation of the antigen binding region. The residues which have been retained as mouse are at positions 2, 3, 48 (?), 60, 84, 85 and 87. Comparison of the murine anti-ICAM 1 and human EU light chain amino acid sequences reveals that the murine and human residues are identical at positions 46, 58 and 71.

HEAVY CHAIN

gH341D has murine CDRs at positions 26-35 (CDR1), 50-56 (CDR2) and94-100B (CDR3). In addition murine residues were used in gH341D at positions 24, 48, 69, 71, 73, 80, 88 and 91. Comparison of the murine anti-ICAM 1 and human EU heavy chain amino acid sequences are identical at positions 23, 49 and 78.

EXAMPLE 5

CDR-Grafting of murine anti-TNFa antibodies

A number of murine anti-TNFa monoclonal antibodies were CDR-grafted substantially as described above in previous examples. These antibodies include the murine monoclonal antibodies designated 61 E71, hTNF1, hTNF3 and 101.4 A brief summary of the CDR-grafting of each of these antibodies is given below.

61E71

A similar analysis as described above (Example 1, Section 12.1.) was done for 61E71 and for the heavy chain 10 residues were identified at 23, 24, 48, 49, 68, 69, 71, 73, 75 and 88 as residues to potentially retain as murine. The human frameworks chosen for CDR-grafting of this antibody, and the hTNF3 and 101.4 antibodies were RE1 for the light chain and KOL for the heavy chain.
Three genes were built, the first of which contained 23, 24, 48, 49, 71 and 73 [gH341(6)] as murine residues. The second gene also had 75 and 88 as murine residues [gH341(8)] while the third gene additionally had 68, 69, 75 and 88 as murine residues [gH341(10)]. Each was co-expressed with gL221, the minimum grafted light chain (CDRs only). The gL221/gH341(6) and gL221/gH341(8) antibodies both bound as well to TNF as murine 61E71. The gL221/gH341(10) antibody did not express and this combination was not taken further.
Subsequently the gL221/gH341(6) antibody was assessed in an L929 cell competition assay in which the antibody competes against the TNF receptor on L929 cells for binding to TNF in solution. In this assay the gL221/gH341(6) antibody was approximately 10% as active as murine 61E71.

hTNF1

hTNF1 is a monoclonal antibody which recognises an epitope on human TNF- . The EU human framework was used for CDR-grafting of both the heavy and light variable domains.

Heavy Chain

In the CDR-grafted heavy chain (ghTNF1) mouse CDRs were used at positions 26-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3). Mouse residues were also used in the frameworks at positions 48, 67, 69, 71, 73, 76, 89, 91, 94 and 108. Comparison of the TNF1 mouse and EU human heavy chain residues reveals that these are identical at positions 23, 24, 29 and 78.

Light Chain

In the CDR-grafted light chain (gLhTNF1) mouse CDRs wre used at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition mouse residues were used in the frameworks at positions 3, 42, 48, 49, 83, 106 and 108. Comparison of the hTNF1 mouse and EU human light chain residues reveals that these are identical at positions 46, 58 and 71.

The grafted hTNF1 heavy chain was co-expressed with the chimeric light chain and the binding ability of the product compared with that of the chimeric light chain/chimeric heavy chain product in a TNF binding assay. The grafted heavy chain product appeared to have binding ability for TNF slightly better than the fully chimeric product.

Similarly, a grafted heavy chain/grafted light chain product was co-expressed and compared with the fully chimeric product and found to have closely similar binding properties to the latter product.

hTNF3

hTNF3 recognises an epitope on human TNF-α. The sequence of hTNF3 shows only 21 differences compared to 61E71 in the light and heavy chain variable regions, 10 in the light chain (2 in the CDRs at positions 50, 96 and 8 in the framework at 1, 19, 40, 45, 46, 76, 103 and 106) and 11 in the heavy chain (3 in the CDR regions at positions 52, 60 and 95 and 8 in the framework at 1, 10, 38, 40, 67, 73, 87 and 105). The light and heavy chains of the 61E71 and hTNF3 chimeric antibodies can be exchanged without loss of activity in the direct binding assay. However 61E71 is an order of magnitude less able to compete with the TNF receptor on L929 cello for TNF-a compared to hTNF3. Based on the 61E71 CDR grafting data gL221 and gH341(+23, 24, 48, 49 71 and 73 as mouse) genes have been built for hTNF3 and tested and the resultant grafted antibody binds well to TNF-a, but competes very poorly in the L929 assay. It is possible that in this case also the framework residues identified for OKT3 programme may improve the competitive binding ability of this antibody.

101.4

101.4 is a further murine monoclonal antibody able to recognise human TNF-a. The heavy chain of this antibody shows good homology to KOL and so the CDR-grafting has been based on RE1 for the light chain and KOL for the heavy chain. Several grafted heavy chain genes have been constructed with conservative choices for the CDR's (gH341) and which have one or a small number of non-CDR residues at positions 73, 78 or 77-79 inclusive, as the mouse amino acids. These have been co-expressed with cL or gL221. In all cases binding to TNF equivalent to the chimeric antibody is seen and when co-expressed with cL the resultant antibodies are able to compete well in the L929 assay. However, with gL221 the resultant antibodies are at least an order of magnitude less able to compete for TNF against the TNF receptor on L929 cells.

Mouse residues at other positions in the heavy chain, for example, at 23 and 24 together or at 76 have been demonstrated to provide no improvement to the competitive ability of the grafted antibody in the L929 assay.

A number of other antibodies including antibodies having specificity for interleukins e.g. IL1 and cancer markers such as carcinoembryonic antigen (CEA) e.g. the monoclonal antibody A5B7 (ref. 21), have been successfully CDR-grafted according to the present invention.

It will be appreciated that the foregoing examples are given by way of illustration only and are not intended to limit the scope of the claimed invention. Changes and modifications may be made to the methods described whilst still falling within the spirit and scope of the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: HUMANISED ANTIBODIES

(iii) NUMBER OF SEQUENCES: 33

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
(EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCCAGATGTT AACTGCTCAC
20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAGGGGCCAG TGGATGGATA GAC
23

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid

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          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

     (v) FRAGMENT TYPE: internal


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

     Leu Glu Ile Asn Arg Thr Val Ala Ala
     1               5

(2) INFORMATION FOR SEQ ID NO: 4:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 943 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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CCTCAGTCAT AATATCCAGA GGACAAATTG TTCTCACCCA GTCTCCAGCA ATCATGTCTG
   120

CATCTCCAGG GGAGAAGGTC ACCATGACCT GCAGTGCCAG CTCAAGTGTA AGTTACATGA
   180

ACTGGTACCA GCAGAAGTCA GGCACCTCCC CCAAAAGATG GATTTATGAC ACATCCAAAC
   240

TGGCTTCTGG AGTCCCTGCT CACTTCAGGG GCAGTGGGTC TGGGACCTCT TACTCTCTCA
   300

CAATCAGCGG CATGGAGGCT GAAGATGCTG CCACTTATTA CTGCCAGCAG TGGAGTAGTA
   360

ACCCATTCAC GTTCGGCTCG GGGACAAAGT TGGAAATAAA CCGGGCTGAT ACTGCACCAA
   420

CTGTATCCAT CTTCCCACCA TCCAGTGAGC AGTTAACATC TGGAGGTGCC TCAGTCGTGT
   480

GCTTCTTGAA CAACTTCTAC CCCAAAGACA TCAATGTCAA GTGGAAGATT GATGGCAGTG
   540

AACGACAAAA TGGCGTCCTG AACAGTTGGA CTGATCAGGA CAGCAAAGAC AGCACCTACA
   600

GCATGAGCAG CACCCTCACG TTGACCAAGG ACGAGTATGA ACGACATAAC AGCTATACCT
   660

GTGAGGCCAC TCACAAGACA TCAACTTCAC CCATTGTCAA GAGCTTCAAC AGGAATGAGT
   720

GTTAGAGACA AAGGTCCTGA GACGCCACCA CCAGCTCCCA GCTCCATCCT ATCTTCCCTT
   780
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CTAAGGTCTT GGAGGCTTCC CCACAAGCGC TTACCACTGT TGCGGTGCTC TAAACCTCCT      840

CCCACCTCCT TCTCCTCCTC CTCCCTTTCC TTGGCTTTTA TGATGCTAAT ATTTGCAGAA      900

AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA                        943
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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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Ile Ser Arg Gly Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser
            20                  25                  30

Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser
        35                  40                  45

Val Ser Tyr Met Asn Trp Tyr Gln Gln Lys Ser Gly Thr Ser Pro Lys
    50                  55                  60

Arg Trp Ile Tyr Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala His
65                  70                  75                  80

Phe Arg Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Gly
                85                  90                  95

Met Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser
            100                 105                 110

Asn Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Asn Arg Ala
        115                 120                 125

Asp Thr Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln Leu
    130                 135                 140

Thr Ser Gly Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr Pro
145                 150                 155                 160

Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln Asn
                165                 170                 175

Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr Tyr
            180                 185                 190

Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg His
        195                 200                 205

Asn Ser Tyr Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser Pro Ile
    210                 215                 220

Val Lys Ser Phe Asn Arg Asn Glu Cys
225                 230
```

(2) INFORMATION FOR SEQ ID NO: 6:

```
(i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 1570 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAATTCCCCT CTCCACAGAC ACTGAAAACT CTGACTCAAC ATGGAAAGGC ACTGGATCTT      60

TCTACTCCTG TTGTCAGTAA CTGCAGGTGT CCACTCCCAG GTCCAGCTGC AGCAGTCTGG     120

GGCTGAACTG GCAAGACCTG GGGCCTCAGT GAAGATGTCC TGCAAGGCTT CTGGCTACAC     180

CTTTACTAGG TACACGATGC ACTGGGTAAA ACAGAGGCCT GGACAGGGTC TGGAATGGAT     240

TGGATACATT AATCCTAGCC GTGGTTATAC TAATTACAAT CAGAAGTTCA AGGACAAGGC     300

CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA GCCTGACATC     360

TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT GATCATTACT GCCTTGACTA     420

CTGGGGCCAA GGCACCACTC TCACAGTCTC CTCAGCCAAA ACAACAGCCC CATCGGTCTA     480

TCCACTGGCC CCTGTGTGTG GAGATACAAC TGGCTCCTCG GTGACTCTAG GATGCCTGGT     540

CAAGGGTTAT TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG     600

TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA GCTCAGTGAC     660

TGTAACCTCG AGCACCTGGC CCAGCCAGTC CATCACCTGC AATGTGGCCC ACCCGGCAAG     720

CAGCACCAAG GTGGACAAGA AAATTGAGCC CAGAGGGCCC ACAATCAAGC CCTGTCCTCC     780

ATGCAAATGC CCAGCACCTA ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA     840

GATCAAGGAT GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT     900

GAGCGAGGAT GACCCAGATG TCCAGATCAG CTGGTTTGTG AACAACGTGG AAGTACACAC     960

AGCTCAGACA CAAACCCATA GAGAGGATTA CAACAGTACT CTCCGGGTGG TCAGTGCCCT    1020

CCCCATCCAG CACCAGGACT GGATGAGTCC CAAGGAGTTC AAATGCAAGG TCAACAACAA    1080

AGACCTCCCA GCGCCCATCG AGAGAACCAT CTCAAAACCC AAAGGGTCAG TAAGAGCTCC    1140

ACAGGTATAT GTCTTGCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC    1200

CTGCATGGTC ACAGACTTCA TGCCTGAAGA CATTTACGTG GAGTGGACCA ACAACGGGAA    1260

AACAGAGCTA AACTACAAGA ACACTGAACC AGTCCTGGAC TCTGATGGTT CTTACTTCAT    1320

GTACAGCAAG CTGAGAGTGG AAAAGAAGAA CTGGGTGGAA AGAAATAGCT ACTCCTGTTC    1380

AGTGGTCCAC GAGGGTCTGC ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTCCGGG    1440

TAAATGAGCT CAGCACCCAC AAAACTCTCA GGTCCAAAGA GACACCCACA CTCATCTCCA    1500

TGCTTCCCTT GTATAAATAA AGCACCCAGC AATGCCTGGG ACCATGTAAA AAAAAAAAAA    1560

AAAGGAATTC                                                           1570

(2) INFORMATION FOR SEQ ID NO: 7:
```

```
 (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 468 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met Glu Arg His Trp Ile Phe Leu Leu Leu Leu Ser Val Thr Ala Gly
1               5                   10                  15

Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg
            20                  25                  30

Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

Thr Arg Tyr Thr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
    50                  55                  60

Glu Trp Ile Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn
65                  70                  75                  80

Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser
                85                  90                  95

Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp
        115                 120                 125

Gly Gln Gly Thr Thr Leu Thr Val Ser Ser Ala Lys Thr Thr Ala Pro
    130                 135                 140

Ser Val Tyr Pro Leu Ala Pro Val Cys Gly Asp Thr Thr Gly Ser Ser
145                 150                 155                 160

Val Thr Leu Gly Cys Leu Val Lys Gly Tyr Phe Pro Glu Pro Val Thr
                165                 170                 175

Leu Thr Trp Asn Ser Gly Ser Leu Ser Ser Gly Val His Thr Phe Pro
            180                 185                 190

Ala Val Leu Gln Ser Asp Leu Tyr Thr Leu Ser Ser Ser Val Thr Val
        195                 200                 205

Thr Ser Ser Thr Trp Pro Ser Gln Ser Ile Thr Cys Asn Val Ala His
    210                 215                 220

Pro Ala Ser Ser Thr Lys Val Asp Lys Lys Ile Glu Pro Arg Gly Pro
225                 230                 235                 240

Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro Ala Pro Asn Leu Leu
                245                 250                 255

Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Ile Lys Asp Val Leu
            260                 265                 270

Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val Val Val Asp Val Ser
        275                 280                 285

Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe Val Asn Asn Val Glu
```

```
    290                 295                 300

Val His Thr Ala Gln Thr Gln Thr His Arg Glu Asp Tyr Asn Ser Thr
305                 310                 315                 320

Leu Arg Val Val Ser Ala Leu Pro Ile Gln His Gln Asp Trp Met Ser
                325                 330                 335

Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys Asp Leu Pro Ala Pro
            340                 345                 350

Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser Val Arg Ala Pro Gln
        355                 360                 365

Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met Thr Lys Lys Gln Val
    370                 375                 380

Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro Glu Asp Ile Tyr Val
385                 390                 395                 400

Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn Tyr Lys Asn Thr Glu
                405                 410                 415

Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met Tyr Ser Lys Leu Arg
            420                 425                 430

Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser Tyr Ser Cys Ser Val
        435                 440                 445

Val His Glu Gly Leu His Asn His His Thr Thr Lys Ser Phe Ser Arg
    450                 455                 460

Thr Pro Gly Lys
465
```

```
(2) INFORMATION FOR SEQ ID NO: 8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 107 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
1               5                   10                  15

Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Lys Ser Gly Thr Ser Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala His Phe Arg Gly Ser
    50                  55                  60

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Gly Met Glu Ala Glu
65                  70                  75                  80

Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95
```

```
        Phe Gly Ser Gly Thr Lys Leu Glu Ile Asn Arg
                    100                 105

    (2) INFORMATION FOR SEQ ID NO: 9:

         (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 108 amino acids
              (B) TYPE: amino acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

        Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
        1               5                   10                  15

        Asp Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr
                    20                  25                  30

        Leu Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile
                35                  40                  45

        Tyr Glu Ala Ser Asn Leu Gln Ala Gly Val Pro Ser Arg Phe Ser Gly
            50                  55                  60

        Ser Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
        65                  70                  75                  80

        Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Gln Ser Leu Pro Tyr
                        85                  90                  95

        Thr Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
                    100                 105

    (2) INFORMATION FOR SEQ ID NO: 10:

         (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 119 amino acids
              (B) TYPE: amino acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

        Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala
        1               5                   10                  15

        Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                    20                  25                  30

        Thr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
                35                  40                  45

        Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Thr Asn Gln Lys Phe
            50                  55                  60

        Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser Thr Ala Tyr
        65                  70                  75                  80
```

```
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Phe Ile Phe Ser Ser Tyr
            20                  25                  30

Ala Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Ile Ile Trp Asp Asp Gly Ser Asp Gln His Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Asp Gly Gly His Gly Phe Cys Ser Ser Ala Ser Cys Phe Gly
            100                 105                 110

Pro Asp Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ser
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala
1               5                   10                  15

Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30
```

```
Thr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

```
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

      Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
      1               5                   10                  15

      Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                  20                  25                  30

      Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
              35                  40                  45

      Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
          50                  55                  60

      Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe
      65                  70                  75                  80

      Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys
                      85                  90                  95

      Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
                  100                 105                 110

      Thr Thr Leu Thr Val Ser Ser
              115

(2) INFORMATION FOR SEQ ID NO: 15:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 119 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein


     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

      Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
      1               5                   10                  15

      Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                  20                  25                  30

      Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
              35                  40                  45

      Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
          50                  55                  60

      Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe
      65                  70                  75                  80

      Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                      85                  90                  95

      Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
                  100                 105                 110

      Thr Thr Leu Thr Val Ser Ser
              115

(2) INFORMATION FOR SEQ ID NO: 16:

     (i) SEQUENCE CHARACTERISTICS:
```

(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Asn Thr Ala Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Ala Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95
```

```
    Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
                100                 105                 110

    Thr Thr Leu Thr Val Ser Ser
            115

(2) INFORMATION FOR SEQ ID NO: 18:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 119 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

    Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
    1               5                   10                  15

    Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                20                  25                  30

    Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
            35                  40                  45

    Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
        50                  55                  60

    Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Asn Thr Leu Phe
    65                  70                  75                  80

    Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                    85                  90                  95

    Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
                100                 105                 110

    Thr Thr Leu Thr Val Ser Ser
            115

(2) INFORMATION FOR SEQ ID NO: 19:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 119 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

    Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
    1               5                   10                  15

    Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                20                  25                  30

    Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
            35                  40                  45
```

```
Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 20:

```
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 119 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 21:

```
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 118 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:
```

```
          Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
          1               5                   10                  15

          Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                      20                  25                  30

          Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
                  35                  40                  45

          Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
              50                  55                  60

          Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
          65                  70                  75                  80

          Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala
                          85                  90                  95

          Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
                      100                 105                 110

          Thr Leu Thr Val Ser Ser
                  115

(2) INFORMATION FOR SEQ ID NO: 22:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 118 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

          Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
          1               5                   10                  15

          Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                      20                  25                  30

          Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
                  35                  40                  45

          Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
              50                  55                  60

          Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
          65                  70                  75                  80

          Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala
                          85                  90                  95

          Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
                      100                 105                 110

          Thr Leu Thr Val Ser Ser
                  115

(2) INFORMATION FOR SEQ ID NO: 23:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 118 amino acids
          (B) TYPE: amino acid
```

```
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein


     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

      Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
      1               5                   10                  15

      Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Tyr Thr Phe Thr Arg Tyr
                  20                  25                  30

      Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
              35                  40                  45

      Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
          50                  55                  60

      Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
      65                  70                  75                  80

      Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala
                      85                  90                  95

      Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
                  100                 105                 110

      Thr Leu Thr Val Ser Ser
              115

(2) INFORMATION FOR SEQ ID NO: 24:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 118 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein


     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

      Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
      1               5                   10                  15

      Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                  20                  25                  30

      Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
              35                  40                  45

      Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
          50                  55                  60

      Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
      65                  70                  75                  80

      Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys Ala
                      85                  90                  95

      Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
                  100                 105                 110
```

```
Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
    50                  55                  60

Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
65                  70                  75                  80

Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys Ala
                85                  90                  95

Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
            100                 105                 110

Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
    50                  55                  60
```

```
Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Asn Thr Ala Phe Leu
65                  70                  75                  80

Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys Ala
                85                  90                  95

Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
            100                 105                 110

Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Phe Ile Phe Ser Ser Tyr
            20                  25                  30

Ala Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Ile Ile Trp Asp Asp Gly Ser Asp Gln His Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Asp Gly Gly His Gly Phe Cys Ser Ser Ala Ser Cys Phe Gly
            100                 105                 110

Pro Asp Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ser
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

```
Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
1               5                   10                  15
```

```
Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Lys Ser Gly Thr Ser Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala His Phe Arg Gly Ser
    50                  55                  60

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Gly Met Glu Ala Glu
65                  70                  75                  80

Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Ser Gly Thr Lys Leu Glu Ile Asn Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

```
Gln Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

```
Gln Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

```
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1               5                   10                  15

 Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
             20                  25                  30

 Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Arg Trp Ile Tyr
         35                  40                  45

 Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
     50                  55                  60

 Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
 65                  70                  75                  80

 Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                 85                  90                  95

 Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
             100                 105

(2) INFORMATION FOR SEQ ID NO: 33:

 (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 108 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: protein


 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1               5                   10                  15

 Asp Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr
             20                  25                  30

 Leu Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile
         35                  40                  45

 Tyr Glu Ala Ser Asn Leu Gln Ala Gly Val Pro Ser Arg Phe Ser Gly
     50                  55                  60

 Ser Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
 65                  70                  75                  80

 Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Gln Ser Leu Pro Tyr
                 85                  90                  95

 Thr Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
             100                 105
```

Claims

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

2. A CDR-grafted heavy chain according to Claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.

3. A CDR-grafted heavy chain according to Claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

4. A CDR-grafted heavy chain according to Claim 2 or 3, comprising donor residues at one, some or all of positions:
1 and 3,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.

6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to Claim 6 comprising donor residues at positions 46 and 47.

8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

9. A CDR-grafted light chain according to Claim 8 comprising donor residues at positions 46, 48, 58 and 71.

10. A CDR-grafted light chain according to Claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

11. A CDR-grafted light chain according to Claim 9 or 10, comprising donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form a potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 if different between donor and acceptor), and
any one or more of 10, 12, 40, 83, 103 and 105.

12. A CDR-grafted light chain according to any one of Claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of Claims 1-5 and at least one CDR-grafted light chain according to any one of Claims 6-12.

14. A CDR-grafted antibody molecule according to Claim 13, which is a site-specific antibody molecule.

15. A CDR-grafted antibody molecule according to Claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.

16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.

17. A DNA sequence which codes for a CDR-grafted heavy chain according to Claim 1 or a CDR-grafted light chain according to Claim 6 or Claim 8.

18. A cloning or expression vector containing a DNA sequence according to Claim 17.

19. A host cell transformed with a DNA sequence according to Claim 17.

20. A process for the production of a CDR-grafted antibody sequence according to Claim 17 in a transformed host cell.

21. A process for producing a CDR-grafted antibody product comprising:
(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to Claim 1;
and/or
(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to Claim 6 or Claim 8;
(c) transfecting a host cell with the or each vector;
and
(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

22. A therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

23. A method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 to a human or animal subject.

1 GAATTCCCAA AGACAAAatg gattttcaag tgcagatttt cagcttcctg
51 ctaatcagtg cctcagtcat aatatccaga ggacaaattg ttctcaccca
101 gtctccagca atcatgtctg catctccagg ggagaaggtc accatgacct
151 gcagtgccag ctcaagtgta agttacatga actggtacca gcagaagtca
201 ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
251 agtccctgct cacttcaggg gcagtgggtc tgggacctct tactctctca
301 caatcagcgg catggaggct gaagatgctg ccacttatta ctgccagcag
351 tggagtagta acccattcac gttcggctcg gggacaaagt tggaaataaa
401 ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
451 agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
501 cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
551 tggcgtcctg aacagttgga ctgatcagga cagcaaagac agcacctaca
601 gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
651 agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
701 gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
751 CCAGCTCCCA GCTCCATCCT ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
801 CCACAAGCGC tTACCACTGT TGCGGTGCTC tAAACCTCCT CCCACCTCCT
851 TCTCCTCCTC CTCCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
901 AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA

Fig. 1(a)

1 MDFQVQIFSF LLISASVIIS RGQIVLTQSP AIMSASPGEK VTMTCSASSS
51 VSYMNWYQQK SGTSPKRWIY DTSKLASGVP AHFRGSGSGT SYSLTISGME
101 AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
151 ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
201 TLTKDEYERH NSYTCEATHK TSTSPIVKSF NRNEC*

Fig. 1(b)

```
   1  GAATTCCCCT CTCCACAGAC ACTGAAAACT CTGACTCAAC ATGGAAAGGC
  51  ACTGGATCTT TCTACTCCTG TTGTCAGTAA CTGCAGGTGT CCACTCCCAG
 101  GTCCAGCTGC AGCAGTCTGG GGCTGAACTG GCAAGACCTG GGGCCTCAGT
 151  GAAGATGTCC TGCAAGGCTT CTGGCTACAC CTTTACTAGG TACACGATGC
 201  ACTGGGTAAA ACAGAGGCCT GGACAGGGTC TGGAATGGAT TGGATACATT
 251  AATCCTAGCC GTGGTTATAC TAATTACAAT CAGAAGTTCA AGGACAAGGC
 301  CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA
 351  GCCTGACATC TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT
 401  GATCATTACT GCCTTGACTA CTGGGGCCAA GGCACCACTC TCACAGTCTC
 451  CTCAGCCAAA ACAACAGCCC CATCGGTCTA TCCACTGGCC CCTGTGTGTG
 501  GAGATACAAC TGGCTCCTCG GTGACTCTAG GATGCCTGGT CAAGGGTTAT
 551  TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG
 601  TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA
 651  GCTCAGTGAC TGTAACCTCG AGCACCTGGC CCAGCCAGTC CATCACCTGC
 701  AATGTGGCCC ACCCGGCAAG CAGCACCAAG GTGGACAAGA AAATTGAGCC
 751  CAGAGGGCCC ACAATCAAGC CCTGTCCTCC ATGCAAATGC CCAGCACCTA
 801  ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA GATCAAGGAT
 851  GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT
 901  GAGCGAGGAT GACCCAGATG TCCAGATCAG CTGGTTTGTG AACAACGTGG
 951  AAGTACACAC AGCTCAGACA CAAACCCATA GAGAGGATTA CAACAGTACT
1001  CTCCGGGTGG TCAGTGCCCT CCCCATCCAG CACCAGGACT GGATGAGTGG
1051  CAAGGAGTTC AAATGCAAGG TCAACAACAA AGACCTCCCA GCGCCCATCG
1101  AGAGAACCAT CTCAAAACCC AAAGGGTCAG TAAGAGCTCC ACAGGTATAT
1151  GTCTTGCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC
1201  CTGCATGGTC ACAGACTTCA TGCCTGAAGA CATTTACGTG GAGTGGACCA
1251  ACAACGGGAA AACAGAGCTA AACTACAAGA ACACTGAACC AGTCCTGGAC
1301  TCTGATGGTT CTTACTTCAT GTACAGCAAG CTGAGAGTGG AAAAGAAGAA
1351  CTGGGTGGAA AGAAATAGCT ACTCCTGTTC AGTGGTCCAC GAGGGTCTGC
1401  ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTCCGGG TAAATGAGCT
1451  CAGCACCCAC AAAACTCTCA GGTCCAAAGA GACACCCACA CTCATCTCCA
1501  TGCTTCCCTT GTATAAATAA AGCACCCAGC AATGCCTGGG ACCATGTAAA
1551   AAAAAAAAAA AAAGGAATTC
```

Fig. 2(a)

OKT 3 HEAVY CHAIN PROTEIN SEQUENCE DEDUCED FROM DNA SEQUENCE

```
  1  MERHWIFLLL LSVTAGVHSQ VQLQQSGAEL ARPGASVKMS CKASGYTFTR
 51  YTMHWVKQRP GQGLEWIGYI NPSRGYTNYN QKFKDKATLT TDKSSSTAYM
101  QLSSLTSEDS AVYYCARYYD DHYCLDYWGQ GTTLTVSSAK TTAPSVYPLA
151  PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGSLSSGVHT FPAVLQSDLY
201  TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC
251  PAPNLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWFV
301  NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMSGKEF KCKVNNKDLP
351  APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV
401  EWTNNGKTEL NYKNTEPVLD SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH
451  EGLHNHHTTK SFSRTPGK*
```

Fig. 2(b)

```
           1                    23                 42
           NN      N              N        N    N
RES TYPE   SBspSPESssBSbSsSssPSPSPsPSsse*s*p*Pi^ISsSe
Okt3vl     QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
REI        DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK
           ? ?
             CDR1   (LOOP)          *******
             CDR1   (KABAT)       ***********


                       56                           85
           N  NN
RES TYPE   *IsiPpIeesesssSBEsePsPSBSSEsPspsPsseesSPePb
Okt3vl     SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
REI        APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT
           ?  ??                       ?  ?
                 *******  CDR2 (LOOP/KABAT)


                         102   108
RES TYPE   PiPIPies**iPIIsPPSPSPSS
Okt3vl     YYCQQWSSNPFTFGSGTKLEINR
REIvl      YYCQQYQSLPYTFGQGTKLQITR
                         ?    ?
               ******       CDR3 (LOOP)
             *********      CRD3 (KABAT)
```

Fig. 3

```
          NN N                 23 26   32 35 N39  43
RES TYPE  SESPs^SBssS^sSSsSpSpSPsPSEbSBssBePiPIpiesss
Okt3h     QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ
KOL       QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
               ?                ??
                                 ******    CDR1 (LOOP)
                                     ***** CDR1 (KABAT)


                   52a      60 65     N N  N   82abc     89
RES TYPE IIeIppp^ssssssss^ps^pSSsbSpseSsSseSp^pSpsSBssS^ePb
Okt3vh   GLEWIGYINPSRGYTNTNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAV
KOL      GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLRPEDTGV
             ??                     ? ?  ?  ?            ?
                ************         CDR2  (LOOP)
                *******************  CDR2  (KABAT)


           92 N                  107    113
RES TYPE  PiPIEissssiiisssbibi*EIPIP*spSBSS
Okt3vh    YYCARYYDDHY.......CLDYWGQGTTLTVSS
KOL       YFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS
               ***************** CRD3 (KABAT/LOOP)
```

Fig. 4

OKT 3 HEAVY CHAIN CDR GRAFTS

1. gh341 and derivatives

```
          1                        26       35  39  43
Okt3vh    QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ
gH341     QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK   JA178
gH341A    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA185

gH341E    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA198
gH341*    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA207
gH341*    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA209
gH341D    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA197
gH341*    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA199
gH341C    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA184

gH341*    QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA203
gH341*    QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA205
gH341B    QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK   JA183
gH341*    QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA204
gH341*    QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA206
gH341*    QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA208
KOL       QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
```

Fig. 5(i)

```
          44   50            65                    83
Okt3vh   GLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLT
gH341    GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR JA178
gH341A   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA185

gH341E   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA198
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTAFLQMDSLR JA207
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTAFLQMDSLR JA209
gH341D   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTLFLQMDSLR JA197
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTLFLQMDSLR JA199
gH341C   GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR JA184

gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA207
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA205
gH341B   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA183
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA204
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA206
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTAFLQMDSLR JA208
KOL      GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLR
```

Fig. 5(ii)

```
          84          95              102        113
Okt3vh    SEDSAVYYCARYYDDHY.......CLDYWGQGTTLTVSS
gH341     PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA178
gH341A    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS    JA185

gH341E    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA198
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA207
gH341D    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA197
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA209
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA199
gH341C    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA184

gH341*    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS    JA203
gH341*    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS    JA205
gH341B    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS    JA183
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA204
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA206
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA208
KOL       PEDTGVYFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS
```

Fig. 5(iii)

OKT3 LIGHT CHAIN CDR GRAFTING

1. gL221 and derivatives

```
            1                      24        34      42
Okt3vl      QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
gL221       DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221A      QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221B      QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221C      DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
REI         DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK

            43      50    56                            85
Okt3vl      SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
gL221       APKLLIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221A      APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221B      APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221C      APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
REI         APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT

            86   91   96          108
Okt3vl      YYCQQWSSNPFTFGSGTKLEINR
gL221       YYCQQWSSNPFTFGQGTKLQITR
gL221A      YYCQQWSSNPFTFGQGTKLQITR
gL221B      YYCQQWSSNPFTFGQGTKLQITR
gL221C      YYCQQWSSNPFTFGQGTKLQITR
REI         YYCQQYQSLPYTFGQGTKLQITR
```

CDR'S ARE UNDERLINED

FRAMEWORK RESIDUES INCLUDED IN THE GENE ARE DOUBLE UNDERLINED

Fig. 6

Fig. 7

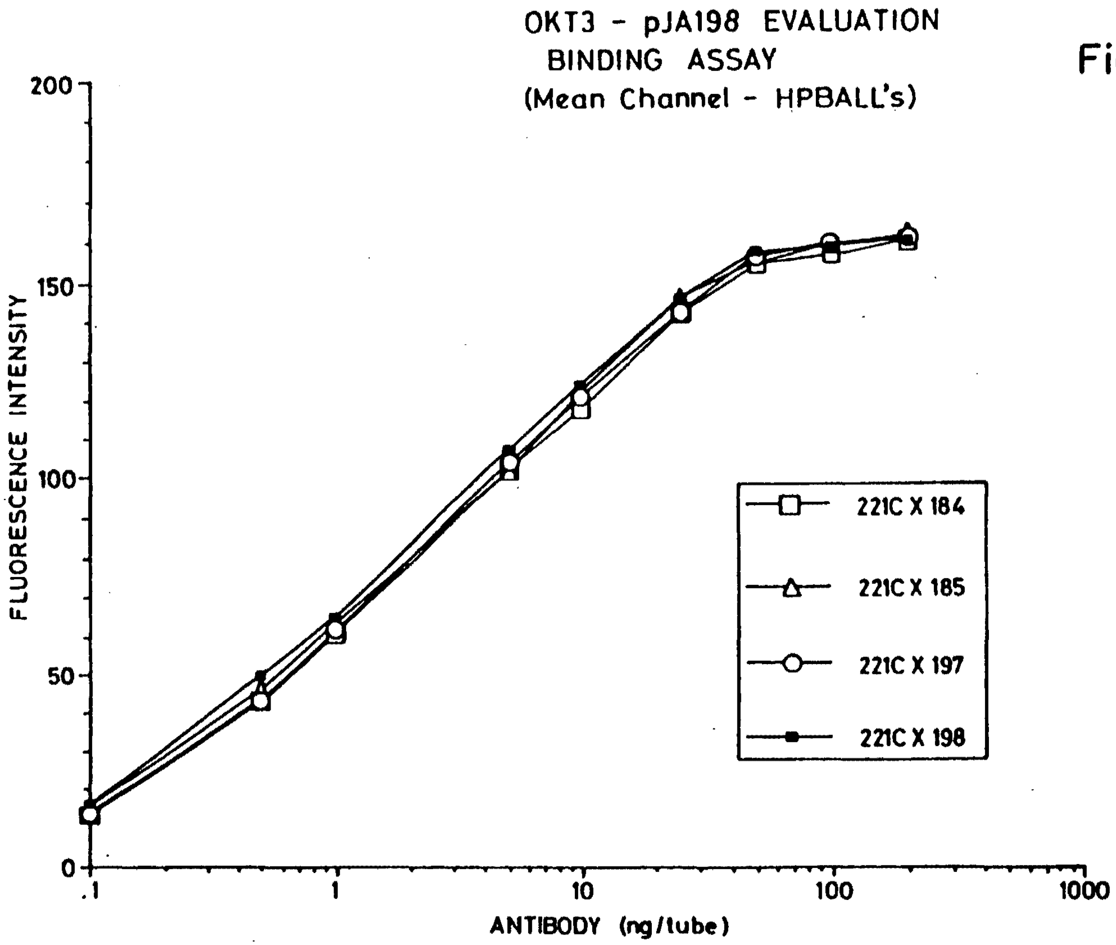
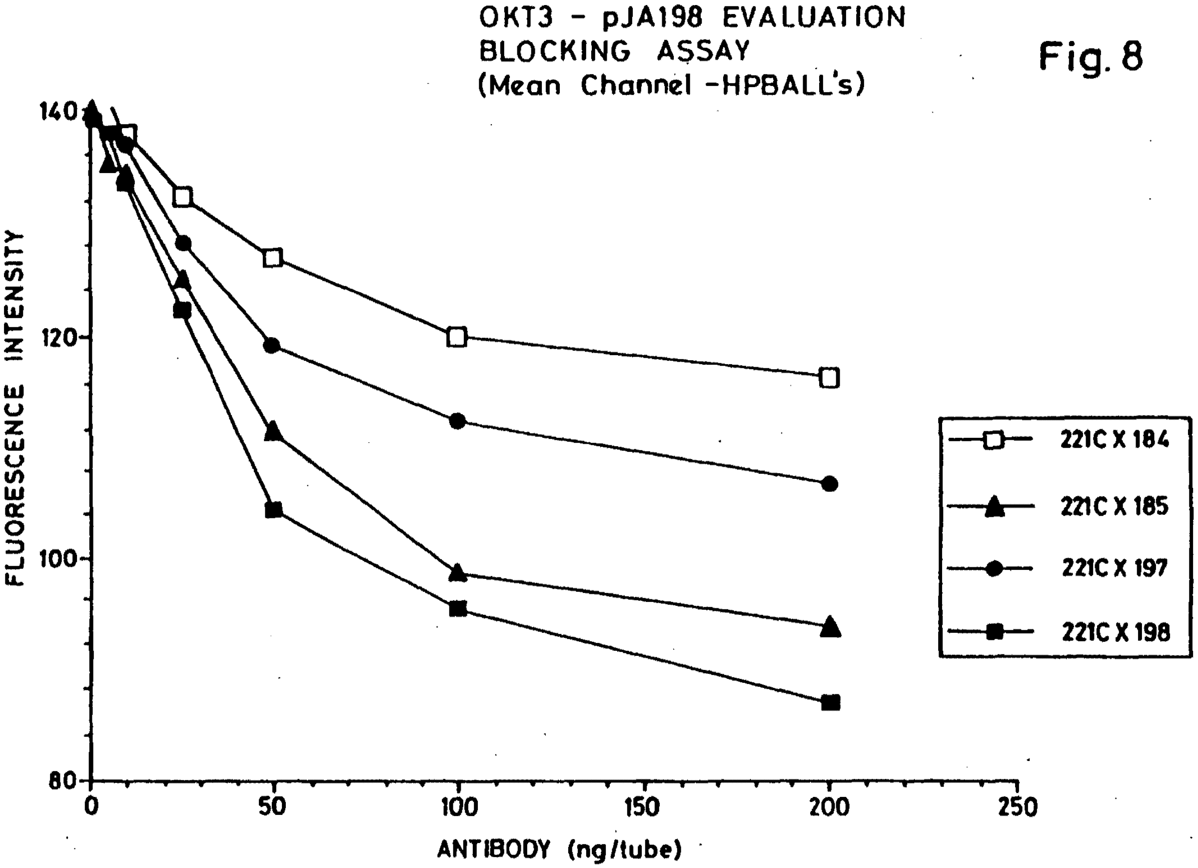

OKT3 - pJA198 EVALUATION
BINDING ASSAY
(Mean Channel - HPBALL's)
FLUORESCENCE INTENSITY
200
150
100
50
0
.1
1
10
100
1000
ANTIBODY (ng/tube)
221C X 184
221C X 185
221C X 197
221C X 198

Fig. 8


OKT3 - pJA198 EVALUATION
BLOCKING ASSAY
(Mean Channel -HPBALL's)
FLUORESCENCE INTENSITY
140
120
100
80
0
50
100
150
200
250
ANTIBODY (ng/tube)
221C X 184
221C X 185
221C X 197
221C X 198

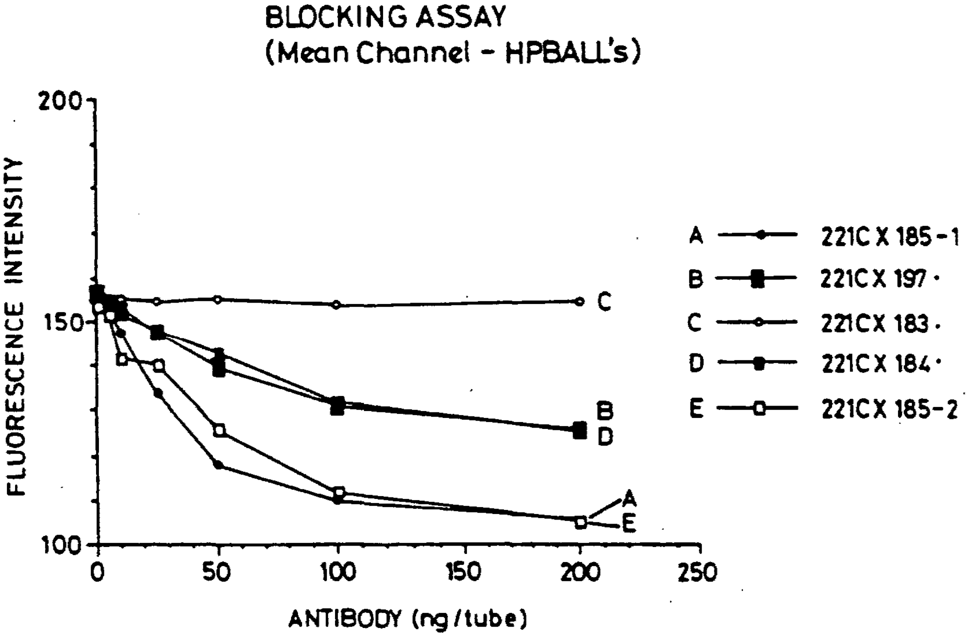
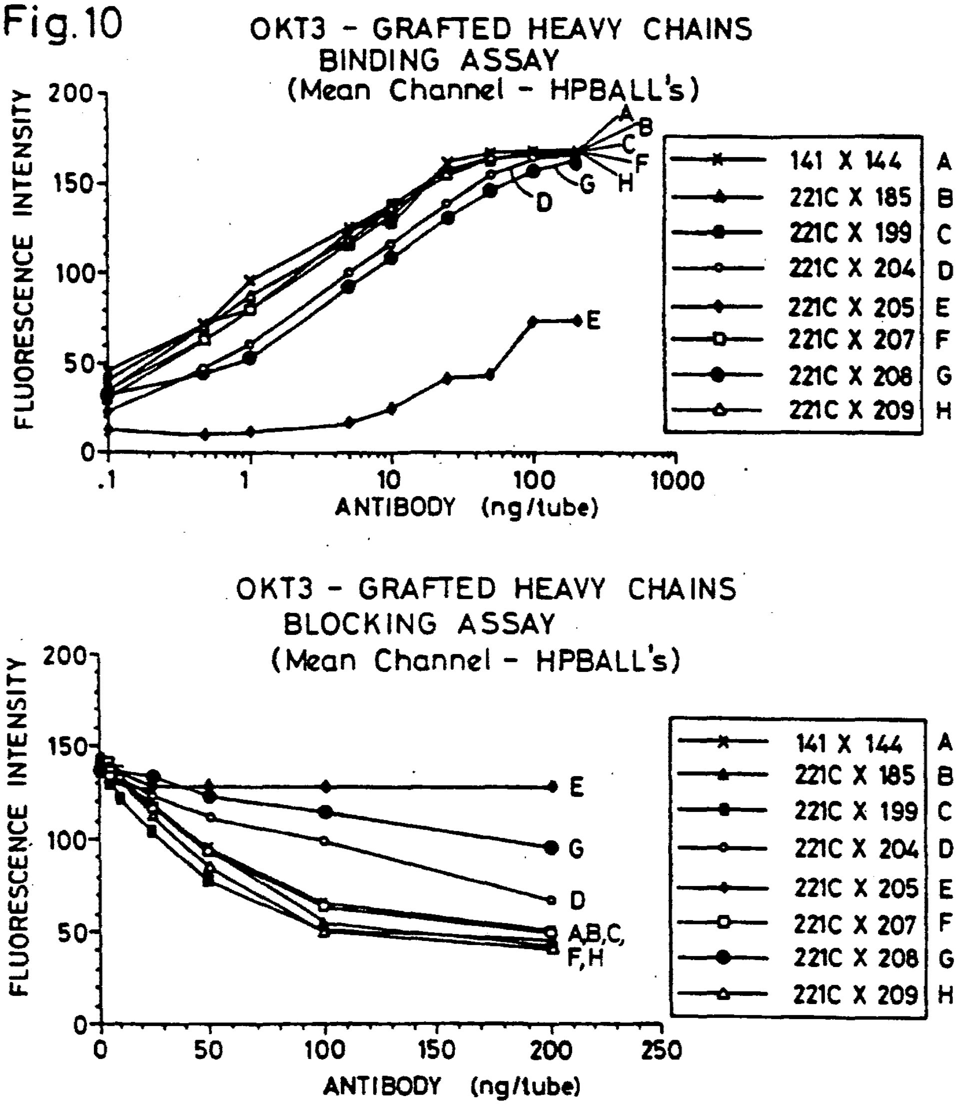

BLOCKING ASSAY
(Mean Channel - HPBALL's)
FLUORESCENCE INTENSITY
200
150
100
0
50
100
150
200
250
ANTIBODY (ng/tube)
A 221C X 185-1
B 221C X 197
C 221CX 183
D 221CX 184
E 221CX 185-2
C
B
D
A
E

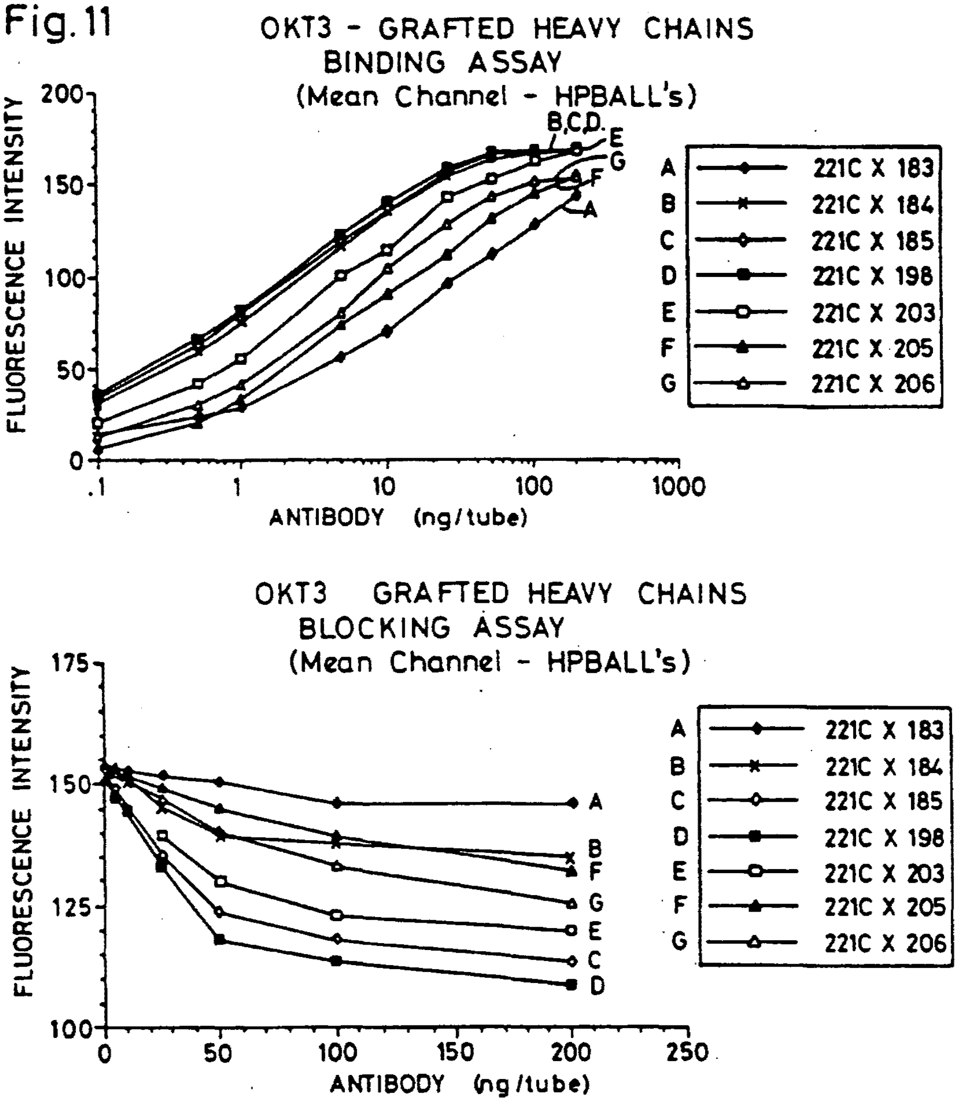
Fig. 9

Fig.10



◆	(205)	_,__,24,48,49,71,73,76,78,88,91,
●	(208)	6,__,24,48,49,71,73,__,78,__,__,
○	(204)	6,__,24,48,49,71,73,76,78,__,__,
■	(199)	6,23,24,48,49,__,__,__,__,__,__,
□	(207)	6,23,24,48,49,71,73,__,78,__,__,
▲	(185)	6,23,24,48,49,71,73,76,78,88,91,
△	(209)	6,23,24,48,49,__,__,__,78,__,__,
×	141 X 144	

Fig. 11



◆	(183)	_,_,_,48,49,71,73,76,78,88,91,
▲	(205)	_,_,24,48,49,71,73,76,78,88,91,
×	(184)	6,23,24,_,_,_,_,_,_,_,_,_,
△	(206)	_,_,24,48,49,71,73,76,78,_,_,
□	(203)	6,_,24,48,49,71,73,76,78,88,91,
◇	(185)	6,23,24,48,49,71,73,76,78,88,91,
■	(198)	6,23,24,48,49,71,73,76,78,_,_,

Fig. 12

OKT3 Competition
Murine Ref Std vs. CDR Grafted OKT3

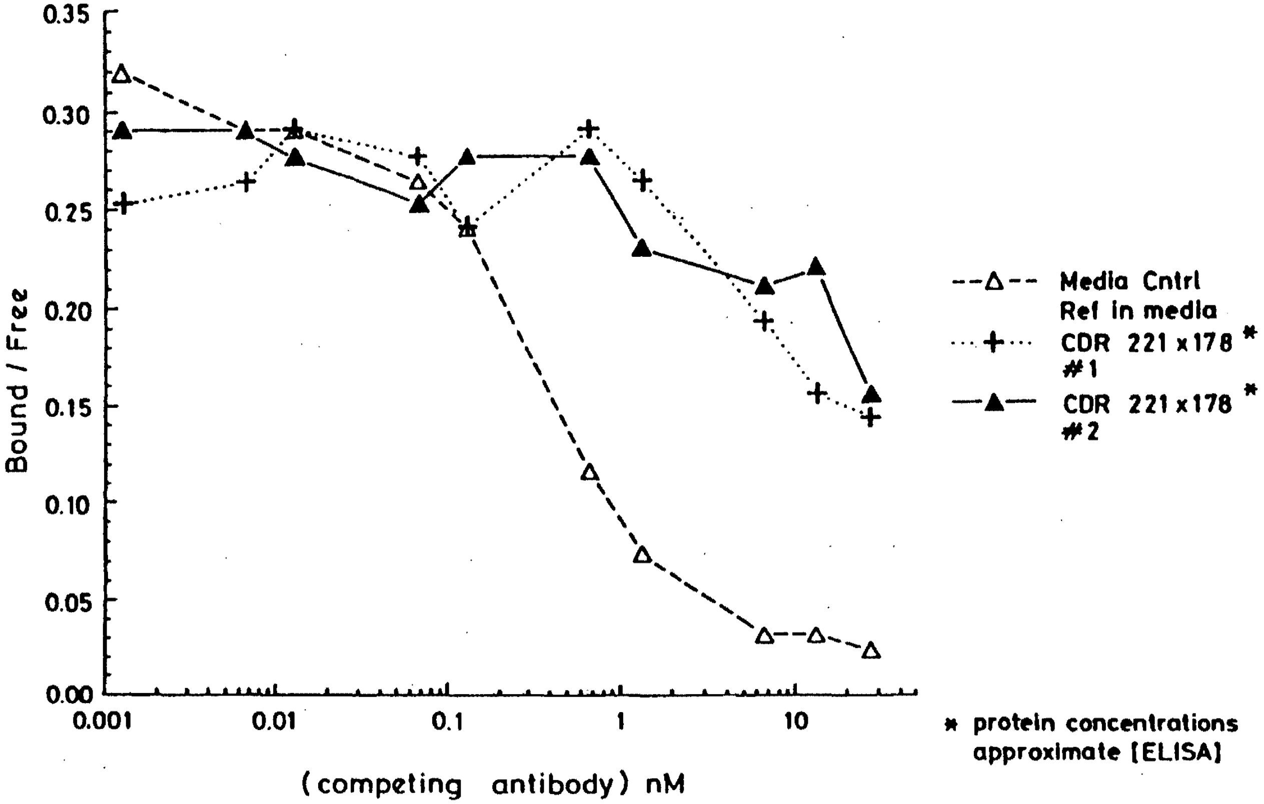
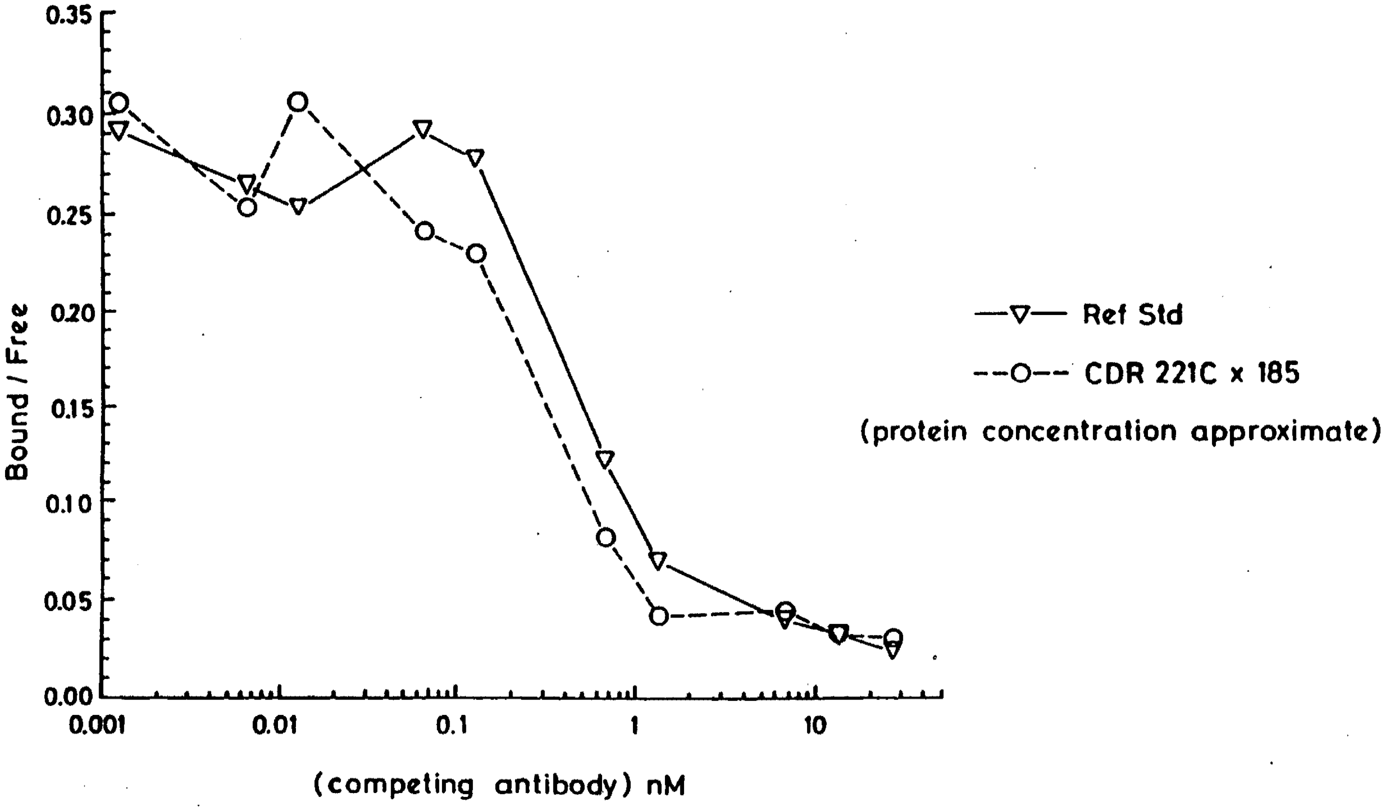


Fig. 13

OKT3 Competition
Murine Ref Std vs. CDR Grafted OKT3



European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number
EP 94 10 4042

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
D,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA vol. 86, no. 24 , 15 December 1989 , WASHINGTON, DC, USA pages 10029 - 10033 C. QUEEN ET AL. 'A humanized antibody that binds to the interleukin 2 receptor.' * the whole document * ---	1-23	C12N15/13 C07K15/28 A61K39/395 G01N33/577
P,X	EP-A-0 403 156 (GENZYME CORPORATION & BEHRINGWERKE) * the whole document * -----	1-23	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)

C12N
C07K
A61K
G01N

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
THE HAGUE	8 June 1994	Nooij, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

EP 94 10 4042

-C-

Remark: Although claim 23
is directed to a method of
treatment of (diagnostic method
practised on) the human/animal body
(Art. 52(4) EPC) the search has been
carried out and based on the
alleged effects of the compound/
composition

PATENT COOPERATION TREATY

13 Rec'd PCT/PTO 17 FEB 1993

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark
Office
Washington, D.C.

in its capacity as elected Office

Date of mailing: 09 February 1993 (09.02.93)	
International application No.: PCT/US92/05126	Applicant's or agent's file reference: 709P1
International filing date: 15 June 1992 (15.06.92)	Priority date: 14 June 1991 (14.06.91)
Applicant: CARTER, Paul, J. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International preliminary Examining Authority on:

07 January 1993 (07.01.93)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer: J. Leitao Telephone No.: (41-22) 730.91.11

Form PCT/IB/331 (July 1992)

5

..TENT COOPERATION TREAT..

PCT

REC'D 23 SEP 1993

WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 709P1	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US 92/05126	International filing date *(day/month/year)* 15/06/1992	Priority date *(day/month/year)* 14/06/1991
International Patent Classification (IPC) or national classification and IPC C12N15/13		
Applicant GENENTECH, INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings amended during international preliminary examination and/or containing rectifications made before this Authority.

These annexes consists of a total of 3 sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 07/01/1993	Date of completion of this report 20.09.93
Name and mailing address of the IPEA/ European Patent Office, Erhardtstrasse 27 W-8000 Munich 2 Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer C. Germinario

Form PCT/IPEA/409 (cover sheet) (July 1992) P20476 (14/06/1993)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern. application No.
PCT/US92/05126

I. Basis of the report

1. This report has been drawn up on the basis of:

[ ] the international application as originally filed.

[x] the description, pages 1-107__________, as originally filed,
pages __________, filed with the demand,
pages __________, filed with the letter of ______,
pages __________, filed with the letter of ______,

[x] the claims, No. 10-17__________, as originally filed,
No. __________, as amended under Article 19,
No. __________, filed with the demand,
No. 1-9, 18, 19__________, filed with the letter of 12.06.93,
No. __________, filed with the letter of ______,

[x] the drawings, sheets/fig 1/9 - 9/9__________, as originally filed,
sheets/fig __________, filed with the demand,
sheets/fig __________, filed with the letter of ______,
sheets/fig __________, filed with the letter of ______.

2. The amendments have resulted in the cancellation of: pages: __________
sheets of drawings/figures No.: __________.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed:

4. Additional observations, if necessary:

Form PCT/IPEA/409 (sheet 1) (July 1992)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern. application No.
PCT/US92/05126

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

[ ] the entire international application,

[x] claims Nos. 17, 18

because:

[x] the said international application, or the said claims Nos. 17, 18 relate to the following subject matter which does not require an international preliminary examination (specify):

Claims 17 is directed to a mere presentation of information, namely the translation of the information inherent in an amino acid sequence into a message or a language readable by the computer.

Claim 18 would appear to be directed to a method of preparing a computer program.
According to Rule 67.1 (V) and (VI) no International Preliminary Examination (thus no preliminary Written Opinion) can be carried out for such a subject matter.

[x] the description, claims or drawings (indicate particular elements below) or said claims Nos. 16 are so unclear that no meaningful opinion could be formed (specify):

Claim 16 represents a novel claim-category; its subject matter is in fact a machine or an apparatus i.e. a computer.
Now an independent claim directed to a machine must cite all the essential technical features necessary to define said machine; the information saved in memory of a computer are not considered a characterizing part of the same. Therefore the subject matter of claim 16 is definitely not at all characterized as requested by Art.

6 PCT (see PCT Guidelines C III 4.4).

[ ] the claims, or said claims Nos. ______________ are so inadequately supported by the description that no meaningful opinion could be formed.

[ ] no international search report has been established for said claims Nos. ______________.

Intern. application No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT PCT/US92/05126

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-9, 12-15, 19	YES
	Claims 10, 11	NO
Inventive Step (IS)	Claims 2, 6-9, 13, 14, 19	YES
	Claims 1, 3-5, 12, 15	NO
Industrial Applicability (IA)	Claims 1-19	YES
	Claims	NO

2. CITATIONS AND EXPLANATIONS

1. The following document is referred to in the present IPER as the closest prior art:

   WO-A-90/07861;

2. This earlier application describes a method for designing humanized antibodies which consists of all the steps a) to g) of the present claim 1.

   More precisely the features under item a) that the amino acid sequences of both donor (import) and acceptor (consensus) antibody are from the variable domain and that the human sequence (acceptor) is a consensus sequence are disclosed at page 10, last two lines and page 11 first lines and page 12 "criterion I.
   Steps under b) and c) are disclosed at page 5 line 8 to 31 and claim 18.
   Steps under items d) to g) are disclosed in claims 19 to 21 and at page 5 line 32 to page 6 line 20 and more in details at page 11 line 19 to page 15 line 2.

Among the three criteria for selecting FR-residues convenient for substitution (item f), criterion 2. is disclosed at page 14 under "criterion IV" and criterion 1. is disclosed at page 14 lines 7 and 8.

2.1 Under "criterion I" at page 12 of the earlier WO application two different options are contemplated for the selection of the acceptor antibody; the first option is based on the homology with the framework of the donor immunoglobulin, the second on the use of a consensus framework from many human antibodies.
The IPEA recognizes that the latter possibility, which corresponds to the present invention, is not further disclosed with details or exemplified.
Therefore the use of a "consensus sequence" as acceptor is not actually an embodiment of the WO-A-90/07861 invention.
For this reason claims 1 to 9, 13 to 15 and 19 are regarded as novel.

2.2. Claims 10 to 12 do not comprise any reference to a consensus sequence as acceptor of the non-human CDR. Therefore the unique feature discriminating between the present invention and the subject matter of the earlier WO application is missing.
It should moreover be noted that the WO-A-90/07861 discloses in details the humanized Eu antibody light chain where the CDRs are replaced by the corresponding CDRs from anti-Tac light chain and where additionally other amino acids in the FR are replace by the corresponding anti-Tac amino acids (see Experimental, page 26, 27; Fig. 2 and explanation of the same at page 7).
From Fig. 2 and explanation of the same is evident that the site 63L of the Eu light chain, which is one of those contemplated by the present claim 10, is replaced by the corresponding amino acid from the anti-Tac light chain (see *).

For this reason claims 10 and 11 are not regarded as novel (Art. 33.2 PCT).

3. Though the WO-A-90/07861 does not discloses in details a consensus sequence, it nevertheless unambiguously suggests the use of a consensus framework from many human antibodies as acceptor sequence (criterion I, page 12). The existence of different criteria (thus not only that based on the homology) for selecting the acceptor sequence is moreover stressed on page 13, line 12, by the sentence ""Regardless of how the acceptor immunoglobulin is chosen..."

Since the reduction to practice of this suggesting is carried out merely by comparing known sequences taken from available collection and designing on paper the requested consensus sequence, the production of said sequence falls within the competence of the skilled person and therefore does not involve <u>per se</u> an inventive merit.

For this reason claims 1 and 15 are not regarded as inventive (Art. 33.3 PCT).

3.1 The ability of the glycosylation sites on the variable domain to influence antigen binding has been known since long time as recognized in the description (see page 3 last paragraph).
Claims 3 and 4 are therefore not regarded as involving an inventive step (Art.33.3 PCT).

3.2 The earlier WO application under "criterion II" at page 13 teaches that "rare residues" in the framework of human acceptor should be replaced by residues from the donor (import) sequence, should said residues (from the donor) be "common" for human sequences at that site.

The interpretation of this teaching by the skilled reader should be that "residues which are highly conserved across all different human antibody types should be conserved".
Therefore also the selecting criterion according to claim 5 is suggested in the earlier WO application . Hence the subject matter of claim 5 is not regarded as involving an inventive step (Art. 33.3 EPC).

4. Claims 2 and claim 19 identify an additional not previously suggested criterion for the selection of the FR-residues suitable for substitution; the subject matter of the two claims is therefore recognized as involving an inventive step.

4.1 Claims 6 to 9 and 13 and 14 are directed to specific embodiments of the invention. Such embodiments do not appear to be disclosed or suggested in the prior art. Said claims are thus recognized as novel and as involving an inventive step.

CLAIMS

WE CLAIM:

1. A method for making a humanized antibody comprising amino acid sequence of a non-human, import antibody and a human antibody, comprising the steps of:
    a. obtaining the amino acid sequences of at least a portion of an import variable domain and of a consensus human variable domain;
    b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human amino variable domain sequences;
    c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;
    d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;
    e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;
    f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:
        1. non-covalently binds antigen directly,
        2. interacts with a CDR; or
        3. participates in the V_L - V_H interface; and
    g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.
2. The method of claim 1, having an additional step of determining if any such non-homologous residues are exposed on the surface of the domain or buried within it, and if the residue is exposed, retaining the consensus residue.
3. The method of claim 1 (or 19,) having the additional steps of searching the import variable domain sequence for glycosylation sites, determining if any such glycosylation site is reasonably expected to affect the antigen binding or affinity of the antibody, and if so, substituting the glycosylation site into the consensus sequence.
4. The method of claim 1 (or 19,) having the additional steps of searching the consensus variable domain sequence for glycosylation sites which are not present at the

corresponding amino acid in the import sequence, and if the glycosylation site is not present in the import sequence, substituting the import amino acid residues for the amino acid residues comprising the consensus glycosylation site.

5. The method of claim 1 or 19, having an additional step which comprises aligning import antibody and consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

6. The method of claim 1, wherein the corresponding consensus antibody residues are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

7. A method comprising providing at least a portion of an import, non-human antibody variable domain amino acid sequence having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus human antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus human antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:
4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

8. The method of claim 7, wherein the substituted residue is the residue found at the corresponding location of the non-human antibody.

9. The method of claim 1 or 7, wherein the consensus human variable domain is a consensus based on human variable domains and additionally variable domains from species other than human.

10. A humanized antibody variable domain having a non-human CDR incorporated into a human antibody variable domain, wherein the improvement comprises

AVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCS
RWGGDGFYAMDVWGQGTLVTVSS

18. A method comprising storing a computer representation of the following amino acid sequence:
    a. DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIY
    AASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFG
    QGTKVEIKRT, or
    b. EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWV
    AVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCS
    RWGGDGFYAMDVWGQGTLVTVSS

19. A method for making a humanized antibody comprising amino acid sequence of a non-human, import antibody and a human antibody, comprising the steps of:
    a. obtaining the amino acid sequences of at least a portion of an import variable domain and of a consensus human variable domain;
    b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human amino variable domain sequences;
    c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;
    d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;
    e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;
    f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:
        1. non-covalently binds antigen directly,
        2. interacts with a CDR; or
        3. participates in the V_L - V_H interface;
    g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence; and
    h. for any non-homologous import antibody amino acid residue, determining if any such non-homologous residue is exposed on the surface of the domain or buried within it, and if the residue is exposed, retaining the consensus residue.

PATENT COOPERATION TREATY

München 21.09.93

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:

BARZ, Peter
P. BARZ & P. WEINHOLD
Siegfriedstrasse 8
D-80803 MÜNCHEN
ALLEMAGNE

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	20.09.93
Applicant's or agent's file reference 709P1	**IMPORTANT NOTIFICATION**

International application No. PCT/US 92/05126	International filing date *(day/month/year)* 15/06/1992	Priority date *(day/month/year)* 14/06/1991

Applicant

GENENTECH, INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/ European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer H.-P. Dietenhofer

Form PCT/IPEA/416 (July 1992) P20473 (15/07/1993)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 709P1	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US 92/05126	International filing date *(day/month/year)* 15/06/1992	Priority date *(day/month/year)* 14/06/1991
International Patent Classification (IPC) or national classification and IPC C12N15/13		
Applicant GENENTECH, INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings amended during international preliminary examination and/or containing rectifications made before this Authority.

These annexes consists of a total of 3 sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 07/01/1993	Date of completion of this report 20.09.93
Name and mailing address of the IPEA/ European Patent Office, Erhardtstrasse 27 W-8000 Munich 2 Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer C. Germinario

Form PCT/IPEA/409 (cover sheet) (July 1992) P20476 (14/06/1993)

Intern. application No.
PCT/US92/05126

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

I. Basis of the report

1. This report has been drawn up on the basis of:

[ ] the international application as originally filed.

[x] the description, pages 1-107 ____________, as originally filed,
pages ____________, filed with the demand,
pages ____________, filed with the letter of ____________,
pages ____________, filed with the letter of ____________,

[x] the claims, No. 10-17 ____________, as originally filed,
No. ____________, as amended under Article 19,
No. q ____________, filed with the demand,
No. 1-9, 18, 19 ____________, filed with the letter of 12.06.93,
No. ____________, filed with the letter of ____________,

[x] the drawings, sheets/fig 1/9 - 9/9 ____________, as originally filed,
sheets/fig ____________, filed with the demand,
sheets/fig ____________, filed with the letter of ____________,
sheets/fig ____________, filed with the letter of ____________.

2. The amendments have resulted in the cancellation of: pages: ____________
sheets of drawings/figures No.: ____________.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed:

4. Additional observations, if necessary:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern. application No.
PCT/US92/05126

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

[ ] the entire international application,

[x] claims Nos. 17, 18

because:

[x] the said international application, or the said claims Nos. 17, 18 relate to the following subject matter which does not require an international preliminary examination (specify):

Claims 17 is directed to a mere presentation of information, namely the translation of the information inherent in an amino acid sequence into a message or a language readable by the computer.

Claim 18 would appear to be directed to a method of preparing a computer program.
According to Rule 67.1 (V) and (VI) no International Preliminary Examination (thus no preliminary Written Opinion) can be carried out for such a subject matter.

[x] the description, claims or drawings (indicate particular elements below) or said claims Nos. 16 are so unclear that no meaningful opinion could be formed (specify):

Claim 16 represents a novel claim-category; its subject matter is in fact a machine or an apparatus i.e. a computer.
Now an independent claim directed to a machine must cite all the essential technical features necessary to define said machine; the information saved in memory of a computer are not considered a characterizing part of the same. Therefore the subject matter of claim 16 is definitely not at all characterized as requested by Art.

Form PCT/IPEA/409 (sheet 2) (July 1992)

6 PCT (see PCT Guidelines C III 4.4).

[ ] the claims, or said claims Nos. ______________________________ are so inadequately supported by the description that no meaningful opinion could be formed.

[ ] no international search report has been established for said claims Nos. ______________________________.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-9, 12-15, 19	YES
	Claims 10, 11	NO
Inventive Step (IS)	Claims 2, 6-9, 13, 14, 19	YES
	Claims 1, 3-5, 12, 15	NO
Industrial Applicability (IA)	Claims 1-19	YES
	Claims	NO

2. CITATIONS AND EXPLANATIONS

1. The following document is referred to in the present IPER as the closest prior art:

   WO-A-90/07861;

2. This earlier application describes a method for designing humanized antibodies which consists of all the steps a) to g) of the present claim 1.

   More precisely the features under item a) that the amino acid sequences of both donor (import) and acceptor (consensus) antibody are from the variable domain and that the human sequence (acceptor) is a consensus sequence are disclosed at page 10, last two lines and page 11 first lines and page 12 "criterion I.
   Steps under b) and c) are disclosed at page 5 line 8 to 31 and claim 18.
   Steps under items d) to g) are disclosed in claims 19 to 21 and at page 5 line 32 to page 6 line 20 and more in details at page 11 line 19 to page 15 line 2.

Among the three criteria for selecting FR-residues convenient for substitution (item f), criterion 2. is disclosed at page 14 under "criterion IV" and criterion 1. is disclosed at page 14 lines 7 and 8.

2.1 Under "criterion I" at page 12 of the earlier WO application two different options are contemplated for the selection of the acceptor antibody; the first option is based on the homology with the framework of the donor immunoglobulin, the second on the use of a consensus framework from many human antibodies.
The IPEA recognizes that the latter possibility, which corresponds to the present invention, is not further disclosed with details or exemplified.
Therefore the use of a "consensus sequence" as acceptor is not actually an embodiment of the WO-A-90/07861 invention.
For this reason claims 1 to 9, 13 to 15 and 19 are regarded as novel.

2.2. Claims 10 to 12 do not comprise any reference to a consensus sequence as acceptor of the non-human CDR. Therefore the unique feature discriminating between the present invention and the subject matter of the earlier WO application is missing.
It should moreover be noted that the WO-A-90/07861 discloses in details the humanized Eu antibody light chain where the CDRs are replaced by the corresponding CDRs from anti-Tac light chain and where additionally other amino acids in the FR are replace by the corresponding anti-Tac amino acids (see Experimental, page 26, 27; Fig. 2 and explanation of the same at page 7).
From Fig. 2 and explanation of the same is evident that the site 63L of the Eu light chain, which is one of those contemplated by the present claim 10, is replaced by the corresponding amino acid from the anti-Tac light chain (see *).

For this reason claims 10 and 11 are not regarded as novel (Art. 33.2 PCT).

3. Though the WO-A-90/07861 does not discloses in details a consensus sequence, it nevertheless unambiguously suggests the use of a consensus framework from many human antibodies as acceptor sequence (criterion I, page 12). The existence of different criteria (thus not only that based on the homology) for selecting the acceptor sequence is moreover stressed on page 13, line 12, by the sentence ""Regardless of how the acceptor immunoglobulin is chosen..."

Since the reduction to practice of this suggesting is carried out merely by comparing known sequences taken from available collection and designing on paper the requested consensus sequence, the production of said sequence falls within the competence of the skilled person and therefore does not involve _per se_ an inventive merit.

For this reason claims 1 and 15 are not regarded as inventive (Art. 33.3 PCT).

3.1 The ability of the glycosylation sites on the variable domain to influence antigen binding has been known since long time as recognized in the description (see page 3 last paragraph).
Claims 3 and 4 are therefore not regarded as involving an inventive step (Art.33.3 PCT).

3.2 The earlier WO application under "criterion II" at page 13 teaches that "rare residues" in the framework of human acceptor should be replaced by residues from the donor (import) sequence, should said residues (from the donor) be "common" for human sequences at that site.

The interpretation of this teaching by the skilled reader should be that "residues which are highly conserved across all different human antibody types should be conserved".
Therefore also the selecting criterion according to claim 5 is suggested in the earlier WO application .
Hence the subject matter of claim 5 is not regarded as involving an inventive step (Art. 33.3 EPC).

4. Claims 2 and claim 19 identify an additional not previously suggested criterion for the selection of the FR-residues suitable for substitution; the subject matter of the two claims is therefore recognized as involving an inventive step.

4.1 Claims 6 to 9 and 13 and 14 are directed to specific embodiments of the invention. Such embodiments do not appear to be disclosed or suggested in the prior art.
Said claims are thus recognized as novel and as involving an inventive step.

ARTICLE 34

M 12.07.93

CLAIMS

WE CLAIM:

1. A method for making a humanized antibody comprising amino acid sequence of a non-human, import antibody and a human antibody, comprising the steps of:
   a. obtaining the amino acid sequences of at least a portion of an import variable domain and of a consensus human variable domain;
   b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human amino variable domain sequences;
   c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;
   d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;
   e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;
   f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:
      1. non-covalently binds antigen directly,
      2. interacts with a CDR; or
      3. participates in the V_L - V_H interface; and
   g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.
2. The method of claim 1, having an additional step of determining if any such non-homologous residues are exposed on the surface of the domain or buried within it, and if the residue is exposed, retaining the consensus residue.
3. The method of claim 1 (or 19,) having the additional steps of searching the import variable domain sequence for glycosylation sites, determining if any such glycosylation site is reasonably expected to affect the antigen binding or affinity of the antibody, and if so, substituting the glycosylation site into the consensus sequence.
4. The method of claim 1 (or 19,) having the additional steps of searching the consensus variable domain sequence for glycosylation sites which are not present at the

ARTICLE 34

M 12.07.93

corresponding amino acid in the import sequence, and if the glycosylation site is not present in the import sequence, substituting the import amino acid residues for the amino acid residues comprising the consensus glycosylation site.

5. The method of claim 1 or 19, having an additional step which comprises aligning import antibody and consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

6. The method of claim 1, wherein the corresponding consensus antibody residues are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

7. A method comprising providing at least a portion of an import, non-human antibody variable domain amino acid sequence having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus human antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus human antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:
4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

8. The method of claim 7, wherein the substituted residue is the residue found at the corresponding location of the non-human antibody.

9. The method of claim 1 or 7, wherein the consensus human variable domain is a consensus based on human variable domains and additionally variable domains from species other than human.

10. A humanized antibody ~~variable~~ domain having a non-human CDR incorporated into a human antibody ~~variable~~ domain, wherein the improvement comprises

SUBSTITUTE SHEET

AVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCS
RWGGDGFYAMDVWGQGTLVTVSS

18. A method comprising storing a computer representation of the following amino acid sequence:
    - a. DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIY
AASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFG
QGTKVEIKRT, or
    - b. EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWV
AVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCS
RWGGDGFYAMDVWGQGTLVTVSS

19. A method for making a humanized antibody comprising amino acid sequence of a non-human, import antibody and a human antibody, comprising the steps of:
    - a. obtaining the amino acid sequences of at least a portion of an import variable domain and of a consensus human variable domain;
    - b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human amino variable domain sequences;
    - c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;
    - d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;
    - e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;
    - f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:
        - 1. non-covalently binds antigen directly,
        - 2. interacts with a CDR; or
        - 3. participates in the V_L - V_H interface;
    - g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence; and
    - h. for any non-homologous import antibody amino acid residue, determining if any such non-homologous residue is exposed on the surface of the domain or buried within it, and if the residue is exposed, retaining the consensus residue.

SUBSTITUTE SHEET

INTERNATIONAL AP[illegible]CATION UNDER THE PATENT COOPERATION TREATY

REQUEST

THE UNDERSIGNED REQUESTS THAT THE PRESENT INTERNATIONAL APPLICATION BE PROCESSED ACCORDING TO THE PATENT COOPERATION TREATY

(The following is to be filled in by the receiving Office)

INTERNATIONAL APPLICATION No. PCT/[illegible]S 92/05126

INTERNATIONAL FILING DATE: 15 JUN 1992

PCT INTERNATIONAL APPLICATION RO/US

(Stamp)
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference (indicated by applicant if desired) 709P1

Box No. I TITLE OF INVENTION

IMMUNOGLOBULIN VARIANTS

Box No. II APPLICANT (WHETHER OR NOT ALSO INVENTOR); DESIGNATED STATES FOR WHICH HE/SHE/IT IS APPLICANT. Use this box for indicating the applicant or, if there are several applicants, one of them. If more than one person (includes, where applicable, a legal entity) is involved, continue in Box No. III.

The person identified in this box is (mark one check-box only): ☐ applicant and inventor* ☒ applicant only

Name *and* address:**

GENENTECH, INC.
460 Point San Bruno Boulevard
South San Francisco, California 94080
United States of America

Telephone number (including area code):	Telegraphic address:	Teleprinter address:
415-225-1000		FAX: 415-952-9881

State of nationality: United States of America State of residence:* United States of America

The person identified in this box is *applicant* for the purposes of (mark one check-box only):
☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the "Supplemental Box"

Box No. III FURTHER APPLICANTS, IF ANY; (FURTHER) INVENTORS, IF ANY; DESIGNATED STATES FOR WHICH THEY ARE APPLICANTS (IF APPLICABLE). A separate sub-box has to be filled in in respect of each person (includes, where applicable, a legal entity). If the following two sub-boxes are insufficient, continue in the "Supplemental Box," (giving there for each additional person the same indications as those requested in the following two sub-boxes) or by using a "continuation sheet."

The person identified in this sub-box is (mark one check-box only): ☒ applicant and inventor* ☐ applicant only ☐ inventor only*

Name *and* address:**

Paul J. CARTER ▲
2074 18th Avenue
San Francisco, California 94116
United States of America

If the person identified in this sub-box is *applicant (or applicant and inventor)*, indicate also:

State of nationality: United Kingdom State of residence:* United States of America

and whether that person is *applicant* for the purposes of (mark one check-box only):
☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the "Supplemental Box"

The person identified in this sub-box is (mark one check-box only): ☒ applicant and inventor* ☐ applicant only ☐ inventor only*

Name *and* address:**

Leonard G. PRESTA ▲
1900 Gough Street, #206
San Francisco, California 94109
United States of America

If the person identified in this sub-box is *applicant (or applicant and inventor)*, indicate also:

State of nationality: United States of America State of residence:* United States of America

and whether that person is *applicant* for the purposes of (mark one check-box only):
☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the "Supplemental Box"

* If the person indicated as "applicant and inventor" or as "inventor only" is not an *inventor* for the purposes of all the designated States, give the necessary indications in the "Supplemental Box."

** Indicate the name of a natural person by giving his/her family name first followed by the given name(s). Indicate the name of a legal entity by its full official designation. In the address, include both the postal code (if any) and the State (name).

*** If residence is not indicated, it will be assumed that the State of residence is the same as the State indicated in the address.

Form PCT/RO/101 (first sheet) (January 1991) See notes on accompanying sheet

▲Surname Underlined By RO/US

PCT/US 92/05126

Sheet number 2

Box No. IV AGENT (IF ANY) OR COMMON REPRESENTATIVE (IF ANY); ADDRESS FOR NOTIFICATIONS (IN CERTAIN CASES). A common representative may be appointed only if there are several applicants and if no agent is or has been appointed; the common representative must be one of the applicants.
The following person (includes, where applicable, a legal entity) is hereby/has been appointed as agent or common representative to act on behalf of the applicant(s) before the competent International Authorities:

Name *and* address, including postal code and country:

If the space below is used instead for an address for notifications, mark here: ☐

Carolyn R. ADLER ▲
GENENTECH, INC.
460 Point San Bruno Boulevard
South San Francisco, California 94080
United States of America

Telephone number (including area code): 415-225-1000

Telegraphic address:

Teleprinter address: FAX: 415-952-9881

Box No. V DESIGNATION OF GROUPS OF STATES OR STATES(1); CHOICE OF CERTAIN KINDS OF PROTECTION OR TREATMENT. The following designations are hereby made (please mark the applicable check-boxes):

Regional Patent

☒ **EP** **European Patent(2):** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, DE Germany, DK Denmark, ES Spain, FR France, GB United Kingdom, GR Greece, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☐ **OA** **OAPI Patent:** Benin, Burkina Faso, Cameroon, Central African Republic, Chad, Congo, Côte d'Ivoire, Gabon, Guinea, Mali, Mauritania, Senegal, Togo, and any other State which is a Contracting State of OAPI and of the PCT; if other OAPI title desired, specify on dotted line(3): ....

National Patent (if other kind of protection or treatment desired, specify on dotted line(3))

- ☐ **AT** Austria(3) ....
- ☒ **AU** Australia(3) ....
- ☐ **BB** Barbados
- ☐ **BG** Bulgaria(3) ....
- ☐ **BR** Brazil(3) ....
- ☒ **CA** Canada
- ☐ **CH and LI** Switzerland and Liechtenstein
- ☐ **CS** Czechoslovakia
- ☐ **DE** Germany(3) ....
- ☐ **DK** Denmark
- ☐ **ES** Spain(3) ....
- ☐ **FI** Finland
- ☐ **GB** United Kingdom
- ☐ **HU** Hungary
- ☒ **JP** Japan(3) ....
- ☐ **KP** Democratic People's Republic of Korea(3) ....
- ☐ **KR** Republic of Korea(3) ....
- ☐ **LK** Sri Lanka
- ☐ **LU** Luxembourg(3) ....
- ☐ **MG** Madagascar
- ☐ **MN** Mongolia(3) ....
- ☐ **MW** Malawi(3) ....
- ☐ **NL** Netherlands
- ☐ **NO** Norway
- ☐ **PL** Poland(3) ....
- ☐ **RO** Romania
- ☐ **SD** Sudan
- ☐ **SE** Sweden
- ☐ **SU** Soviet Union
- ☒ **US** United States of America(3) ....
  continuation-in-part,

Space reserved for designating States (for the purposes of a national patent) which have become party to the PCT after the issuance of this sheet:

....

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(1) The applicant's choice of the order of designations may be indicated by marking the check-boxes with sequential arabic numerals (see also the "Notes to Box No. V").

(2) The selection of particular States for a European patent can be made upon entering the national (regional) phase before the European Patent Office (see also the "Notes to Box No. V").

(3) If another kind of protection or a title of addition or, in the United States of America, treatment as a continuation or a continuation-in-part is desired, specify according to the instructions given in the "Notes to Box No. V."

Form PCT/RO/101 (second sheet) (January 1992) See notes on accompanying sheet

▲Surname Underlined By RO/US

Sheet number ....3.... PCT/US 92/05126

Supplemental Box. Use this box [illegible]ollowing cases:

(i) *if more than three persons are involved as applicants and/or inventors;* in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III;

(ii) *if, in Box No. II or any of the sub-boxes of Box No. III, the indication "the States indicated in the 'Supplemental Box,'" is checked;* in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State or States (or EP or OA, if applicable) for the purposes of which he/she/it is applicant;

(iii) *if, in Box No. II or any of the sub-boxes of Box No. III, a person indicated as "applicant and inventor" or "inventor only" is not inventor for the purposes of all designated States or for the purposes of the United States of America;* in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor and, next to such name, the State or States (or EP or OA, if applicable) for the purposes of which the named person is inventor;

(iv) *if there is more than one agent and their addresses are not the same;* in such case, write "Continuation of Box No. IV" and indicate for each additional agent the same type of information as required in Box No. IV;

(v) *if, in Box No. V, the name of any country (or OAPI) is accompanied by the indication "patent of addition," "certificate of addition," or "inventor's certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part";* in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of parent title or filing of parent application;

(vi) *if there are more than three earlier applications whose priority is claimed;* in such case, indicate "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;

(vii) *if, in any of the Boxes, the space is insufficient to furnish all the information;* in such case, write "Continuation of Box No...." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient;

(viii) *if the applicant intends to claim, in respect of any designated Office, the benefit of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty;* in such case, write "Statement Concerning Non-prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below.

Continuation Box IV

Also: BUTING, Walter E., DREGER, Ginger R., FITTS, Renee A., HASAK, Janet E., HENSLEY, Max D., GLAISTER, Debra J., RAINES, Stephen, WINTER, Daryl B.

All of: GENENTECH, INC.
460 Point San Bruno Boulevard
South San Francisco, California 94080
United States of America

Continuation Box V

United States of America Application Serial Number 715,272 filed 14 June 1991.

(14.06.91)

If this Supplemental Box is not used, this sheet need not be included in the Request.

Box No. VI PRIORITY CLAIM (IF ANY). The priority of the following earlier application(s) is hereby claimed:

Country (country in which it was filed if national application; one of the countries for which it was filed if regional or international application)	Filing Date (day, month, year)	Application No.	Office of filing (fill in only if the earlier application is an international application or a regional application)
(1) US	14 June 1991 (14.06.91)	715,272	
(2)			
(3)			

(Letter codes may be used to indicate country and/or Office of filing)

When the earlier application was filed with the Office which, for the purposes of the present international application, is the receiving Office, the applicant may, *against payment of the required fee*, ask the following:

☒ the receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the above-mentioned earlier application/of the earlier applications identified above by the numbers (insert the applicable numbers) (1)

Box No. VII EARLIER SEARCH (IF ANY). Fill in where a search (international, international-type or other) by the International Searching Authority has already been requested (or completed) and the said Authority is now requested to base the international search, to the extent possible, on the results of the said earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request.

International application number or number and country (or regional Office) of other application:

International/regional/national filing date:

Date of request for search:

Number (if available) given to search request:

Box No. VIII SIGNATURE OF APPLICANT(S) OR AGENT

Carolyn R. Adler

(Stephen RAINES - Vice President
Intellectual Property,
GENENTECH, INC.)

(Carolyn R. ADLER, Agent for
Paul J. CARTER, Leonard G. PRESTA)

If the present Request form is signed on behalf of any applicant by an agent, a separate power of attorney appointing the agent and signed by the applicant is required. If in such case it is desired to make use of a general power of attorney (deposited with the receiving Office), a copy thereof must be attached to this form.

Box No. IX CHECK LIST (To be filled in by the Applicant)

This international application contains the following number of sheets:

1.	request	4	sheets
2.	description	107	sheets
3.	claims	5	sheets
4.	abstract	1	sheets
5.	drawings	9	sheets
	Total	126	sheets

Figure number 2 of the drawings (if any) is suggested to accompany the abstract for publication.

This international application as filed is accompanied by the items marked below:

1. ☒ separate signed power of attorney To be filed within 30 days
2. ☐ copy of general power of attorney
3. ☒ priority document(s) (see Box No. VI) ordered above
4. ☐ receipt of the fees paid or revenue stamps
5. ☐ cheque for the payment of fees
6. ☒ request to charge deposit account 07-0630
7. ☒ other document (specify) Transmittal Sheet, Fee Calculation Sheet

(The following is to be filled in by the receiving Office)

1. Date of actual receipt of the purported international application: 13 Rec'd PCT/PC 15 JUN 1992
2. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
3. Date of timely receipt of the required corrections under Article 11 of the PCT:
4. Drawings ☐ Received ☐ No Drawings

(The following is to be filled in by the International Bureau)

Date of receipt of the record copy:

THIS SHEET IS NOT PART OF AND DOES NOT COUNT AS A SHEET OF THE INTERNATIONAL APPLICATION

APPLICANT
GENENTECH, INC. et al.

This column for use by receiving Office

INTERNATIONAL APPLICATION NUMBER
(to be filled in by the receiving Office)
PCT/US 92/05126

DATE STAMP OF RECEIVING OFFICE

FEE CALCULATION SHEET[1]

FEES SUBMITTED OR TO BE CHARGED TO DEPOSIT ACCOUNT

I. **TRANSMITTAL FEE**[2]	190	T	190
II. **SEARCH FEE**[3]	1320	S	1,320

International search to be effected by EP
(Please indicate, but only if the applicant has the choice between two or more International Searching Authorities, the name of the Authority to which the international application is to be transmitted. Note that the amount of the search fee depends on the identity of the International Searching Authority.)

III. **INTERNATIONAL FEE**[4]

BASIC FEE[5]

Indicate the number of SHEETS contained in the international application 126.

first 30 sheets	525	b₁	525
remaining 96 sheets × 10 =	960	b₂	960
Add amounts entered in boxes b₁ and b₂ and enter total in box B. This figure is the amount of the BASIC FEE	1485	B	1,485

DESIGNATION FEES[5]

Indicate the number of NATIONAL PATENTS which have been sought and multiply by the amount of the designation fee. 4 × 127 =	508	d₁	508
Indicate the number of REGIONAL PATENTS which have been sought and multiply by the amount of the designation fee. 1 × 127 =	127	d₂	127
Add amounts entered in boxes d₁ and d₂ and enter total in box D (if that total exceeds the figure which corresponds to the amount of the designation fee multiplied by ten, enter the latter figure in Box D)[6]. This figure is the amount of the DESIGNATION FEES	635	D	635
Add amounts entered in boxes B and D, and enter total in box I. This figure is the total amount of the INTERNATIONAL FEE	2120	I	2,120

IV. **TOTAL OF PRESCRIBED FEES SUBMITTED OR TO BE CHARGED TO DEPOSIT ACCOUNT**

Add amounts entered in boxes T, S and I, and enter total in the TOTAL box. This figure is the amount of the PRESCRIBED FEES SUBMITTED OR TO BE CHARGED TO DEPOSIT ACCOUNT	3630. TOTAL	$3,630

THE APPLICANT MAY PAY THE PRESCRIBED FEES BY [CHEQUE, POSTAL MONEY ORDER, BANK DRAFT, CASH, REVENUE STAMPS, COUPONS, ETC.]. PAYMENT SHOULD BE MADE IN THE PRESCRIBED CURRENCY TO THE [ACCOUNT OF, ACCOUNT INDICATED BELOW OF, ORDER OF] THE RECEIVING OFFICE. PAYMENT MAY ALSO BE MADE BY AUTHORIZATION TO CHARGE A DEPOSIT ACCOUNT AT THE RECEIVING OFFICE IF THE LATTER HAS A DEPOSIT ACCOUNT SYSTEM.

DEPOSIT ACCOUNT AUTHORIZATION[7]

☒ The RO/ US is hereby authorized to charge the total fees indicated above to my deposit account.
☒ The RO/ US is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.
☒ The RO/ US is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account Number: 07-0630
Date: 12 June 1992
Signature:

Form PCT/RO/101 (Annex) (January 1991)

See notes on reverse side

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 709P1	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US92/05126	International filing date *(day/month/year)* 15/06/92	(Earliest) Priority Date *(day/month/year)* 14/06/91

Applicant

GENENTECH, INC. et al.

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☒ **Certain claims were found unsearchable** (see Box I).

2. ☐ **Unity of invention is lacking** (see Box II).

3. ☐ The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - ☐ filed with the international application.
   - ☐ furnished by the applicant separately from the international application,
     - ☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - ☐ Transcribed by this Authority

4. With regard to the **title**,
   - ☐ the text is approved as submitted by the applicant.
   - ☒ the text has been established by this Authority to read as follows:

   METHOD FOR MAKING HUMANIZED ANTIBODIES.

5. With regard to the **abstract**,
   - ☒ the text is approved as submitted by the applicant.
   - ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. 2
   - ☐ as suggested by the applicant.
   - ☐ because the applicant failed to suggest a figure.
   - ☐ because this figure better characterizes the invention.
   - ☐ None of the figures.

Form PCT/ISA/210 (first sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/05126

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 C12N15/13; C12P21/08; C07K13/00; C12N5/10
G06F15/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; G06F

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	JOURNAL OF MOLECULAR BIOLOGY vol. 215, 1990, ACADEMIC PRESS pages 175 - 182 Tramontano, Anna; Chothia, Cyrus; Lesk, Arthur M. 'Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins' cited in the application See the whole document, especially paragraph 7 ---	1-12,15
Y	WO,A,9 007 861 (PROTEIN DESIGN LABS, INC.) 26 July 1990 See pages 1-6; 9-25 --- -/--	1-12,15

° 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

2 **IV. CERTIFICATION**

Date of the Actual Completion of the International Search 07 OCTOBER 1992	Date of Mailing of this International Search Report 02. 11. 92
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer NAUCHE S.A.

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

See notes on accompanying sheet

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	NATURE. vol. 342, December 1989, LONDON GB pages 877 - 883 Chothia, Cyrus; Lesk, Arthur M.; Tramontano, Anna; Levitt, Michael; Smith-Gill, Sandra J.; Air, Gillian; Sheriff, Steven; Padlan, 'Conformations of immunoglobulin hypervariable region' cited in the application See the whole document, especially 'Discussion' ---	1-12,15
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 89, May 1992, WASHINGTON US pages 4285 - 4289 Carter, Paul et al. 'Humanization of an anti-p185HER2 antibody for human cancer therapy.' see the whole document -----	1-15

INTERNATIONAL S[illegible]RCH REPORT

International application No.
PCT[illegible]S 92/ 05126

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

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

1. ☒ Claims Nos.: 17-18
because they relate to subject matter not required to be searched by this Authority, namely:
see PCT-Rule 39.1(iv)

2. ☐ Claims Nos.:
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. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ 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 claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9205126

SA 61838

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 07/10/92
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
WO-A-9007861	26-07-90	AU-A-	5153290	13-08-90
		CA-A-	2006865	28-06-90
		EP-A-	0451216	16-10-91

EPO FORM P0479

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

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark
Office
Washington, D.C.

in its capacity as elected Office

PCT

NOTIFICATION CONCERNING DOCUMENT TRANSMITTED

Date of mailing: 24 September 1993 (24.09.93)	
International application No.: PCT/US92/05126	International filing date: 15 June 1992 (15.06.92)
Applicant: GENENTECH, INC. et al	

The International Bureau transmits herewith the following documents and number thereof:

________ copy of the international preliminary examination report and annexes (Article 36(3)(a))

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorised officer: B. Fitzgerald Telephone No.: (41-22) 730.91.11

Form PCT/IB/310 (July 1992)

DO/US WORKSHEET

U.S. Appl. No. 08/1[illegible] 206

Internationa[illegible]ppl No. US92/5126

Application filed by: ☐ 20 months -- ☑ 30 months

INTERNATIONAL APPLICATION PAPERS IN THE APPLICATION FILE:

☑ International application (RECORD COPY)
☐ Article 19 amendments
☒ PCT/IB/331
☒ PCT/IPEA/409 IPER (PCT/IPEA/416 on front)
☒ Annexes to 409
☐ Priority document(s) No.______
☐ INTERNATIONAL APPLICATION ON DOUBLE SIDED PAPER (COPIES MADE)

☒ Request form PCT/RO/101
☐ PCT/IB/302
☒ PCT/ISA/210-Search Report
☑ Search Report references
☐ Other 310

RECEIPTS FROM THE APPLICANT: (other than checked above)

☑ Basic National Fee (paid or authorized to charge)
Translation of international application as filed:
  ☐ Description
  ☐ Claims
  ☐ Words in the drawing figure(s)
  ☐ Article 19 amendments
  ☐ Annexes to 409
☑ Oath / Declaration
☑ DNA diskette

☑ Preliminary amendment(s) filed 17 NOV 1993
☐ Information Disclosure Statement
☐ Assignment document
☐ Power of attorney/Change of address
☐ Substitute specification
☐ Verified small status claim
☐ Other ______

Notes: ARTICLE 34 NOT ENTITY
CLAIMS are incomplete.

35 U.S.C. 371 - Receipt of Request (PTO-1390)	17 NOV 1993
Date acceptable oath / declaration received	17 NOV 1993
Date complete 35 U.S.C 371 requirements met	17 NOV 1993
102(e) Date	17 NOV 1993
Date of completion of DO/EO 906 - Notification of Missing 102(e) Requirements	
Date of completion of DO/EO 907 - Notification of Acceptance for 102(e) date	
Date of completion of DO/EO 911 - Application accepted under 35 U.S.C. 1.11	
Date of completion of DO/EO 905 - Notification of Missing Requirements	
Date of completion of DO/EO 916 - Notification of Defective Response	
Date of completion of DO/EO 903 - Notification of Acceptance	29 MAR 1994
Date of completion of DO/EO 909 - Notification of Abandonment	

WIPO Publication
Publ.ication No.
WO/______
Publication Date
______
Publication Language
______
Not Published
☐ U.S. only
Designated
☐ EP request

Screening done by: HANNIE

May 1993

EXPRESS MAIL LABEL: HB214759258US
MAILED: 17 NOVEMBER 1993
Attorney's Docket No. 709P1

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office and citizenship are as stated below next to my name,

I believe I am the original and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR MAKING HUMANIZED ANTIBODIES

the specification of which (check only one item below):

__ is attached hereto.

__ was filed as United States application Serial No. ______________ on ______________________
and was amended on ______________________ (if applicable.).

x was filed as PCT international application Number PCT/US92/05126 on 15 JUNE 1992 and was amended under PCT Article 19 on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of such of the claims of this application is not disclosed in that/those priori application(s) in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information a defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Attorney's Docket No. 709P1

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Continued)

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS *(Check one)*		
U.S. Application Number	U.S. Filing Date	Patented	Pending	Abandoned
07/715,272	14 June 1991		x	ABN

PCT APPLICATIONS DESIGNATING THE U.S.

PCT Application No.	PCT Filing Date	U.S. Serial Numbers	Patented	Pending	Abandoned

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

9

Carolyn R. Adler - Reg. No. 32,324	Sean A. Johnston - Reg. No. 35,910
Renee A. Fitts - Reg. No. 35,136	Dennis G. Kleid - Reg. No. 32,037
Walter E. Buting - Reg. No. 23,092	Janet E. Hasak - Reg. No. 28,616
Ginger R. Dreger - Reg. No. 33,055	Stephen Raines - Reg. No. 25,912
Daryl B. Winter - Reg. No. 32,637	

Send correspondence to

Genentech, Inc.
Attn: Janet E. Hasak
460 Point San Bruno Boulevard
South San Francisco, CA 94080-4990
Telephone: (415) 225-1896

I hereby declare that all statements made herein of my own knowledge 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 willful false statements may jeopardize the validity of the application or any patent issue thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein at accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

Full name of sole or first inventor
13 > Paul J. Carter

Inventor's signature Paul J. Carter — Date 10/14/93

Residence
2074 18th Avenue, San Francisco, CA 94116 CA

Citizenship
United Kingdom

Post Office Address
2074 18th Avenue, San Francisco, CA 94116

Full name of second or joint inventor, if any
Leonard G. Presta

Second Inventor's signature Leonard G. Presta — Date 10/14/93

Residence
1900 Gough Street, #206, San Francisco, CA 94109 CA

Citizenship
U.S.A.

Post Office Address
1900 Gough Street, #206, San Francisco, CA 94109

08/143,394
08/090,902
08/222,274
08/144,206

08/146206

05 Rec'd PCT/PTO 17 NOV 1993

1806

PATENT DOCKET 709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#6

RECEIVED
JUN 1 1994
APPLICATION DIVISION

In re Application of)
Paul J. Carter et al.)
Serial No. To Be Assigned)
Filed: 17 November 1993)
For: METHOD OF MAKING HUMANIZED ANTIBODIES)

Art Unit: To Be Assigned
Examiner: To Be Assigned

6-13-94

460 Point San Bruno Boulevard
South San Francisco, CA 94080-4990
(415) 225-1896

PRELIMINARY AMENDMENT

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

RECEIVED
JUN 10 1994
GROUP 1800

6/13/94

Sir:

In the Specification:

Please amend the specification by inserting after page 76 the attached Sequence Listing as pages 77-92.

Please further amend the specification by renumbering pages 95-99 to be pages 93-97.

Remarks

This amendment is prepared for the purposes of introducing a substitute sequence listing into the application. In accordance with 37 C.F.R. § 1.821(f), I hereby state that this Sequence Listing is submitted in paper copy and in computer-readable copy, and that the content of these copies are the same, without adding any new matter.

Early entry of these amendments is requested. The inventors submit that this application is now in compliance with the requirement of 37 C.F.R. §1.821-1.825.

Respectfully submitted,
GENENTECH, INC.

Janet E. Hasak

Janet E. Hasak
Reg. No. 28,616

Date: November 17, 1993

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Paul J. Carter
Leonard G. Presta

(ii) TITLE OF INVENTION: Method for Making Humanized Antibodies

(iii) NUMBER OF SEQUENCES: 25

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: patin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 07/715272
(B) FILING DATE: 14-JUN-1991

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Hasak, Janet E.
(B) REGISTRATION NUMBER: 28,616
(C) REFERENCE/DOCKET NUMBER: 709P1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/225-1896
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(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
```

a1

(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 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: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 bases
(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 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTTGATCTC CAGCTTGGTA CCHSCDCCGA A 31

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(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 bases
(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 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 bases
(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 bases
(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 bases
(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 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
CTATACCTCC CGTCTGCATT CTGGAGTCCC 30
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Arg
                20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys
                35                  40                  45

Leu Leu Ile Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser
                50                  55                  60

Lys Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
                65                  70                  75
```

```
Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
                 80                  85                  90

Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly Gly Thr Lys Leu Glu
                 95                 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

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: 129 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 Arg Phe Thr Ile Ser Lys Ala Thr Leu
                65                      70                  75

Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr Leu Met Glu Leu Leu
                80                      85                  90

Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg
                95                      100                 105

Ser Gly Tyr Tyr Gly Asp Ser Asp Trp Tyr Phe Asp Val Trp Gly
                110                     115                 120

Ala Gly Thr Thr Val Thr Val Ser Ser
                125                 129
```

(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:

a1

```
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
```

a1

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 557 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
His His Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
  1             5                   10                  15

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr
                20                  25                  30

Phe Thr Glu Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala
                35                  40                  45

Thr Ala Thr Gly Val His Ser Glu Val Gln Leu Val Glu Ser Gly
                50                  55                  60

Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala
                65                  70                  75

Thr Ser Gly Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met Arg
                80                  85                  90

Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn Pro
                95                  100                 105

Lys Asn Gly Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg Phe
                110                 115                 120

Thr Ile Ser Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln Met
                125                 130                 135

Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
                140                 145                 150

Trp Arg Gly Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp Val
                155                 160                 165

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys
                170                 175                 180

Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser
                185                 190                 195

Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro
                200                 205                 210

Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
                215                 220                 225

Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
                230                 235                 240

Leu Ser Ser Val Val Thr Val Thr Ser Ser Asn Phe Gly Thr Gln
                245                 250                 255

Thr Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val
                260                 265                 270
```

```
Asp Lys Thr Val Glu Arg Lys Cys Cys Val Thr Cys Pro Pro Cys
                275                 280                 285

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
                290                 295                 300

Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val
                305                 310                 315

Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
                320                 325                 330

Glu Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val
                335                 340                 345

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
                350                 355                 360

Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp
                365                 370                 375

Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Met Glu Val His
                380                 385                 390

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe
                395                 400                 405

Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp Leu Asn
                410                 415                 420

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ala
                425                 430                 435

Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu
                440                 445                 450

Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys
                455                 460                 465

Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
                470                 475                 480

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
                485                 490                 495

Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe
                500                 505                 510

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
                515                 520                 525

Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
                530                 535                 540

Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
                545                 550                 555
```

(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
```

UNITED STATES DEPARTMENT OF COMMERCE
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#2

U.S. APPLICATION NO.		FIRST NAMED APPLICANT	ATTY. DOCKET NO.
08/146,206		CARTER P	709P1

5611

CAROLYN R. ADLER
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO, CALIFORNIA 94080

INTERNATIONAL APPLICATION NO.
PCT/US92/05126

I.A. FILING DATE	PRIORITY DATE
06/15/92	06/14/91

DATE MAILED: 04/04/94

NOTIFICATION OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

1. The applicant is hereby advised that the United States Patent and Trademark Office in its capacity as ☐ a Designated Office (37 CFR 1.494), ☑ an Elected Office (37 CFR 1.495), has determined that the above identified international application has met the requirements of 35 U.S.C. 371, and is **ACCEPTED** for national patentability examination in the United States Patent and Trademark Office.

2. The United States Application Number assigned to the application is shown above and the relevant dates are:

17 NOV 1993 — 35 U.S.C. 102(e) DATE

17 NOV 1993 — DATE OF RECEIPT OF 35 U.S.C. 371 REQUIREMENTS

3. ☑ A request for immediate examination under 35 U.S.C. 371(f) was received on 17 NOV 1993 and the application will be examined in turn.

4. The following items have been received:
- ☑ U.S. Basic National Fee.
- ☑ Copy of the international application in:
  - ☐ a non-English language.
  - ☑ English.
- ☐ Translation of the international application into English.
- ☑ Oath or Declaration of inventors(s) for DO/EO/US.
- ☐ Copy of Article 19 amendments. ☐ Translation of Article 19 amendments into English.
  - The Article 19 amendments ☐ have ☐ have not been entered.
- ☑ The International Preliminary Examination Report in English and its Annexes, if any.
- ☐ Translation of Annexes to the International Preliminary Examination Report into English.
  - The Annexes ☐ have ☐ have not been entered.
- ☑ Preliminary amendment(s) filed 17 NOV 1993 and ________.
- ☐ Information Disclosure Statement(s) filed ________ and ________.
- ☑ Assignment document.
- ☐ Power of Attorney and /or Change of Address.
- ☐ Substitute specification filed ________.
- ☐ Verified Statement Claiming Small Entity Status.
- ☐ Priority Document.
- ☑ Copy of the Search Report ☑ and copies of the references cited therein.
- ☑ Other: ARTICLE 34 AMENDMENTS NOT ENTITY, CLAIMS ARE INCOMPLETE.

A Filing Receipt (PTO-103X) will be issued for the present application in due course. Once the Filing Receipt has been received, send all correspondence to the Group Art Unit designated thereon.

Applicant is reminded that any communication to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above. (37 CFR 1.5)

Telephone: (703) 3053165

FORM PCT/DO/EO/903 (May 1993)

UNITED STATES PATENT & TRADEMARK OFFICE
Washington, D.C. 20231

REQUEST FOR PATENT FEE REFUND

1 Date of Request: 29 MAR94

2 Serial/Patent # 08/146206

	3 Please refund the following fee(s):	4 PAPER NUMBER	5 DATE FILED	6 AMOUNT
✓	Filing	1	17NOV93	$ 172.00
	Amendment			$
	Extension of Time			$
	Notice of Appeal/Appeal			$
	Petition			$
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	Cert of Correction/Terminal Disc.			$
	Maintenance			$
	Assignment			$
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7 TOTAL AMOUNT OF REFUND $ 172.00

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9 07--0630

10 REASON:

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EPO SEARCH

11 REFUND REQUESTED BY:

TYPED/PRINTED NAME: M PERSON

TITLE: Paralegal/Specialist

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PHONE: 3053737

OFFICE: PCT

****

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FORM PTO 1577
(01/90)

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CL #4

PAGE: 1

RAW SEQUENCE LISTING
PATENT APPLICATION ***US/08/146,206***

DATE: 04/15/94
TIME: 12:13:19

INPUT SET: S2658.raw

```
                              SEQUENCE LISTING

(1)    General Information:

   (i) APPLICANT: Paul J. Carter
                  Leonard G. Presta

  (ii) TITLE OF INVENTION: Method for Making Humanized Antibodies

 (iii) NUMBER OF SEQUENCES: 25

  (iv) CORRESPONDENCE ADDRESS:
       (A) ADDRESSEE: Genentech, Inc.
       (B) STREET: 460 Point San Bruno Blvd
       (C) CITY: South San Francisco
       (D) STATE: California
       (E) COUNTRY: USA
       (F) ZIP: 94080

   (v) COMPUTER READABLE FORM:
       (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
       (B) COMPUTER: IBM PC compatible
       (C) OPERATING SYSTEM: PC-DOS/MS-DOS
       (D) SOFTWARE: patin (Genentech)

  (vi) CURRENT APPLICATION DATA:
       (A) APPLICATION NUMBER:
       (B) FILING DATE:
       (C) CLASSIFICATION:

 (vii) PRIOR APPLICATION DATA:
       (A) APPLICATION NUMBER: 07/715272
       (B) FILING DATE: 14-JUN-1991

(viii) ATTORNEY/AGENT INFORMATION:
       (A) NAME: Hasak, Janet E.
       (B) REGISTRATION NUMBER: 28,616
       (C) REFERENCE/DOCKET NUMBER: 709P1

  (ix) TELECOMMUNICATION INFORMATION:
       (A) TELEPHONE: 415/225-1896
       (B) TELEFAX: 415/952-9881
       (C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:23:

   (i) SEQUENCE CHARACTERISTICS:
-->    (A) LENGTH: 557 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:
```

only 552 are shown, Please review discrepancy.

```
His His Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
  1               5                  10                  15

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr
                 20                  25                  30

Phe Thr Glu Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala
                 35                  40                  45

Thr Ala Thr Gly Val His Ser Glu Val Gln Leu Val Glu Ser Gly
                 50                  55                  60

Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala
                 65                  70                  75

Thr Ser Gly Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met Arg
                 80                  85                  90

Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn Pro
                 95                 100                 105

Lys Asn Gly Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg Phe
                110                 115                 120

Thr Ile Ser Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln Met
                125                 130                 135

Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
                140                 145                 150

Trp Arg Gly Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp Val
                155                 160                 165

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys
                170                 175                 180

Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser
                185                 190                 195

Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro
                200                 205                 210

Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
                215                 220                 225

Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
                230                 235                 240

Leu Ser Ser Val Val Thr Val Thr Ser Ser Asn Phe Gly Thr Gln
                245                 250                 255

Thr Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val
                260                 265                 270
```

```

Asp Lys Thr Val Glu Arg Lys Cys Cys Val Thr Cys Pro Pro Cys
                275                 280                 285

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
                290                 295                 300

Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val
                305                 310                 315

Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
                320                 325                 330

Glu Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val
                335                 340                 345

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
                350                 355                 360

Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp
                365                 370                 375

Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Met Glu Val His
                380                 385                 390

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe
                395                 400                 405

Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp Leu Asn
                410                 415                 420

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ala
                425                 430                 435

Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu
                440                 445                 450

Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys
                455                 460                 465

Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
                470                 475                 480

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
                485                 490                 495

Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe
                500                 505                 510

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
                515                 520                 525

Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
```

```
                    530                       535                       540

Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
                    545                       550                       555

```

only 552 are shown.

SEQUENCE VERIFICATION REPORT
PATENT APPLICATION *US/08/146,206*

DATE: 04/15/94
TIME: 12:13:37

INPUT SET: S2658.raw

Line	Error	Original Text
696	Entered (557) and Calc. Seq. Length (552) differ	(A) LENGTH: 557 amino acids

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	ATTY. DOCKET NO./TITLE
08/146,206	11/17/93	CARTER	P 709P1

03A1/0502

CAROLYN R. ADLER
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO, CALIFORNIA 94080

0000

DATE MAILED: 05/02/94

NOTICE TO FILE MISSING PARTS OF APPLICATION FILING DATE GRANTED

An Application Number and Filing Date have been assigned to this application. However, the items indicated below are missing. The required items and fees identified below must be timely submitted **ALONG WITH THE PAYMENT OF A SURCHARGE** for items 1 and 3-6 only of $__________ for large entities or $__________ for small entities who have filed a verified statement claiming such status. The surcharge is set forth in 37 CFR 1.16(e).

If all required items on this form are filed within the period set below, the total amount owed by applicant as a ☐ large entity, ☐ small entity (verified statement filed), is $__________.

Applicant is given **ONE MONTH FROM THE DATE OF THIS LETTER, OR TWO MONTHS FROM THE FILING DATE** of this application, **WHICHEVER IS LATER,** within which to file all required items and pay any fees required above to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

1. ☐ The statutory basic filing fee is: ☐ missing ☐ insufficient. Applicant as a ☐ large entity ☐ small entity, must submit $__________ to complete the basic filing fee.
2. ☐ Additional claim fees of $__________ as a ☐ large entity, ☐ small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.
3. ☐ The oath or declaration:
   ☐ is missing.
   ☐ does not cover items omitted at time of execution.

   An oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date is required.
4. ☐ The oath or declaration does not identify the application to which it applies. An oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
5. ☐ The signature to the oath or declaration is: ☐ missing; ☐ a reproduction; ☐ by a person other than the inventor or a person qualified under 37 CFR 1.42, 1.43, or 1.47. A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
6. ☐ The signature of the following joint inventor(s) is missing from the oath or declaration:

   ____________________ An oath or declaration listing the names of all inventors and signed by the omitted inventor(s), identifying this application by the above Application Number and Filing Date, is required.
7. ☐ The application was filed in a language other than English. Applicant must file a verified English translation of the application and a fee of $__________ under 37 CFR 1.17(k), unless this fee has already been paid.
8. ☐ A $__________ processing fee is required for returned checks. (37 CFR 1.21(m)).
9. ☐ Your filing receipt was mailed in error because check was returned without payment.
10. ☑ The application does not comply with the Sequence Rules. See attached Notice to Comply with Sequence Rules 37 CFR 1.821-1.825.
11. ☐ Other.

Direct the response and any questions about this notice to C. Barnes, Application Processing Division, Special Processing and Correspondence Branch (703) 308-1202.

A copy of this notice MUST be returned with the response.

FORM PTO-1533 (REV. 5-93)

OFFICE COPY

Application No. 08/146 206

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 CFR 1.821 - 1.825 for the following reason(s):

☐ 1. This application clearly fails to comply with the requirements of 37 CFR 1.821 - 1.825. Applicant's attention is directed to these regulations, published at 1114 OG 29, May 15, 1990 and at 55 FR 18230, May 1, 1990.

☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 CFR 1.821(c).

☐ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e).

☑ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 CFR 1.822 and/or 1.823, as indicated on the attached copy of the marked-up "Raw Sequence Listing."

☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A substitute computer readable form must be submitted as required by 37 CFR 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable form of the "Sequence Listing" as required by 37 CFR 1.821(e).

☐ 7.
Other: ____________________

Applicant must provide:

☐ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing"

☐ An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification

☑ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 CFR 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d)

For questions regarding compliance with these requirements, please contact:

For Rules Interpretation, call (703) 308-1123
For CRF submission help, call (703) 308-4212
For PatentIn software help, call (703) 557-0400

Please return a copy of this notice with your response.

dlg. #110

MAIL ROOM
27 JUN 6 1994
PAT. & TRADEMARK OFF.

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	ATTY. DOCKET NO./TITLE
08/146,206	11/17/93	CARTER	P 709P1

03A1/0502

CAROLYN R. ADLER
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO, CALIFORNIA 94080

0000

DATE MAILED: 05/02/94

NOTICE TO FILE MISSING PARTS OF APPLICATION
FILING DATE GRANTED

An Application Number and Filing Date have been assigned to this application. However, the items indicated below are missing. The required items and fees identified below must be timely submitted **ALONG WITH THE PAYMENT OF A SURCHARGE** for items 1 and 3-6 only of $__________ for large entities or $__________ for small entities who have filed a verified statement claiming such status. The surcharge is set forth in 37 CFR 1.16(e).

If all required items on this form are filed within the period set below, the total amount owed by applicant as a ☐ large entity, ☐ small entity (verified statement filed), is $__________.

Applicant is given **ONE MONTH FROM THE DATE OF THIS LETTER, OR TWO MONTHS FROM THE FILING DATE** of this application, **WHICHEVER IS LATER,** within which to file all required items and pay any fees required above to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

1. ☐ The statutory basic filing fee is: ☐ missing ☐ insufficient. Applicant as a ☐ large entity ☐ small entity, must submit $__________ to complete the basic filing fee.

2. ☐ Additional claim fees of $__________ as a ☐ large entity, ☐ small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

3. ☐ The oath or declaration:
   ☐ is missing.
   ☐ does not cover items omitted at time of execution.

   An oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date is required.

4. ☐ The oath or declaration does not identify the application to which it applies. An oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.

5. ☐ The signature to the oath or declaration is: ☐ missing; ☐ a reproduction; ☐ by a person other than the inventor or a person qualified under 37 CFR 1.42, 1.43, or 1.47. A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.

6. ☐ The signature of the following joint inventor(s) is missing from the oath or declaration:

   ____________________ An oath or declaration listing the names of all inventors and signed by the omitted inventor(s), identifying this application by the above Application Number and Filing Date, is required.

7. ☐ The application was filed in a language other than English. Applicant must file a verified English translation of the application and a fee of $__________ under 37 CFR 1.17(k), unless this fee has already been paid.

8. ☐ A $__________ processing fee is required for returned checks. (37 CFR 1.21(m)).

9. ☐ Your filing receipt was mailed in error because check was returned without payment.

10. ☑ The application does not comply with the Sequence Rules. See attached Notice to Comply with Sequence Rules 37 CFR 1.821-1.825.

11. ☐ Other.

Direct the response and any questions about this notice to____________________, Application Processing Division, Special Processing and Corresp dence Branch (703) 308-1202.

A copy of this notice M rned with the response.

FORM PTO-1533 (REV. 5-93)

H RESPONSE

Application No.

NOTICE TO COMPLY WITH [illegible]UIREMENTS FOR PATENT APPL[illegible]ATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 CFR 1.821 - 1.825 for the following reason(s):

☐ 1. This application clearly fails to comply with the requirements of 37 CFR 1.821 - 1.825. Applicant's attention is directed to these regulations, published at 1114 OG 29, May 15, 1990 and at 55 FR 18230, May 1, 1990.

☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 CFR 1.821(c).

☐ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e).

☑ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 CFR 1.822 and/or 1.823, as indicated on the attached copy of the marked-up "Raw Sequence Listing."

☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A substitute computer readable form must be submitted as required by 37 CFR 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable form of the "Sequence Listing" as required by 37 CFR 1.821(e).

☐ 7.
Other: ____________________

Applicant must provide:

☐ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing"

☐ An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification

☑ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 CFR 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d)

For questions regarding compliance with these requirements, please contact:

For Rules Interpretation, call (703) 308-1123
For CRF submission help, call (703) 308-4212
For PatentIn software help, call (703) 557-0400

Please return a copy of this notice with your response.

MAIL ROOM
21 JUN 6 1994
PAT. & TRADEMARK OFF.

PATENT DOCKET 709P1

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: Unknown

Examiner: Unassigned

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on

June 2, 1994
(Date of Deposit)

Elisa R. Hamby
Name of Depositing Party

Signature of Depositing Party

6/2/94
Date of Signature

CERTIFICATE RE: SEQUENCE LISTING

BOX SEQUENCE
Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I hereby state that the Sequence Listing submitted with this application is submitted in paper copy and a computer-readable diskette, and that the content of the paper and computer readable copies are the same.

A copy of a document pursuant to 37 C.F.R. § 10.9(b) is attached as proof of the authorization of the undersigned to prosecute the above-mentioned application. The original of this document is on file in the Office of Enrollment and Discipline.

Respectfully submitted,

GENENTECH, INC.

Date: 6/2/94

By:
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

MAIL ROOM
21 JUN 6 1994
PAT. & TRADEMARK OFF.

12C

~~#14~~

1805

PATENT DOCKET 709P1

#7
Adams
6-14

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: Unknown Examiner: Unassigned

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on
June 2, 1994
(Date of Deposit)
Elisa R. Hamby
Name of Depositing Party
Elisa R Hamby
Signature of Depositing Party
6/2/94
Date of Signature

6/14/94

AMENDMENT

BOX SEQUENCE
Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

This is responsive to the Notice to File Missing Parts of Application - Filing Date Granted and Notice to Comply with Sequence Rules Pursuant to 37 CFR 1.821-1.825, mailed 2 May 1994. The due date for this response is 2 June 1994. This response is timely filed.

Please amend the application as follows:

IN THE SPECIFICATION

Please amend the specification by replacing the original Sequence Listing pages 77-94 with the attached corrected Sequence Listing as pages 77-94.

REMARKS

An error in the original Sequence Listing filed 11/17/93 was found in SEQ ID NO:23 in that there claimed to be 557 amino acids, and only 552 residues are shown. This error has been corrected and now corresponds to Figure 6A and the sequence entitled "pH52-8.0". Another error was found in SEQ ID NO:19 which has also been corrected and now corresponds to Figure 5 (lower panel) and the sequence entitled "muxCD3".

The inventors submit that this application is now in compliance with the requirements of 37 CFR 1.821-1.825, and respectfully request further processing of this application.

A copy of a document pursuant to 37 C.F.R. § 10.9(b) is attached as proof of the authorization of the undersigned to prosecute the above-mentioned application. The original of this document is on file in the Office of Enrollment and Discipline.

Respectfully submitted,
GENENTECH, INC.

Date: 6/2/94

By: WMLee
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

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: 25

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

F.

B1

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: patin (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: Hasak, Janet E.
(B) REGISTRATION NUMBER: 28,616
(C) REFERENCE/DOCKET NUMBER: 709P1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/225-1896
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(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
```

B1

(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 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
```

B1

(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 bases
(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 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTTGATCTC CAGCTTGGTA CCHSCDCCGA A 31

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(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 bases
(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 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 bases
(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 bases
(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 bases
(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 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
CTATACCTCC CGTCTGCATT CTGGAGTCCC 30
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Arg
                 20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys
                 35                  40                  45

Leu Leu Ile Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser
                 50                  55                  60

Lys Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
                 65                  70                  75

Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
                 80                  85                  90

Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly Gly Thr Lys Leu Glu
                 95                 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

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
```

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(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

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
```

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(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
```

B1 Cont

AU 1806

PAGE: 1

RAW SEQUENCE LISTING
PATENT APPLICATION *US/08/146,206A*

DATE: 06/14/94
TIME: 17:05:48

INPUT SET: S8112.raw

#8

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: 25

  (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Genentech, Inc.
        (B) STREET: 460 Point San Bruno Blvd
        (C) CITY: South San Francisco
        (D) STATE: California
        (E) COUNTRY: USA
        (F) ZIP: 94080

   (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: patin (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: Hasak, Janet E.
        (B) REGISTRATION NUMBER: 28,616
        (C) REFERENCE/DOCKET NUMBER: 709P1

  (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 415/225-1896
        (B) TELEFAX: 415/952-9881
        (C) TELEX: 910/371-7168

(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 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: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

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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 bases
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:


TCCGATATCC AGCTGACCCA GTCTCCA 27
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SEQUENCE VERIFICATION REPORT
PATENT APPLICATION *US/08/146,206A*

DATE: 06/14/94
TIME: 17:06:53

INPUT SET: S8112.raw

Line	Error	Original Text
27	Wrong application Serial Number	(A) APPLICATION NUMBER: 08/146206

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1994
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PAT. & TRADEMARK OFF.

#9 A/17

PATENT DOCKET 709P1

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: 1804

Examiner: Unassigned

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on
June 24, 1994
(Date of Deposit)
Elisa R. Hamby
Name of Depositing Party
Elisa R. Hamby
Signature of Depositing Party
6/24/94
Date of Signature

RECEIVED
JUL 11 1994
GROUP 1800

RECEIVED
JUL 06 1994
APPLICATION

REQUEST FOR A CORRECTED FILING RECEIPT

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Attached is a copy of the Official Filing Receipt received from the PTO in the above application for which issuance of a corrected filing receipt is respectfully requested. Please make the correction as follows: Under "CONTINUING DATA..." please add --WHICH IS A CIP OF 07/715,272 06/14/91--; and please correct the title to read --METHOD FOR MAKING HUMANIZED ANTIBODIES--.

The correction is not due to any error by applicant and no fee is believed to be due. However, in the event that the Patent Office determines that fees are due in connection with the filing of this document, we hereby authorize the Commissioner to charge such fees to our Deposit Account No. 07-0630.

A copy of a document pursuant to 37 C.F.R. § 10.9(b) is attached as proof of the authorization of the undersigned to prosecute the above-mentioned application. The original of this document is on file in the Office of Enrollment and Discipline.

Respectfully submitted,
GENENTECH, INC.

Date: 6/24/94

By: W. M. Lee
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

17/22/94

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JUN 28
1994
64
PAT. & TRADEMARK OFF.

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

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 assignee 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 December 9, 1994: (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) Wendy M. Lee ceases to remain or reside in the United States on an 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, 1994

Cameron Weiffenbach

Cameron Weiffenbach, Director
Office of Enrollment and Discipline

PTO-103X
(Rev. 7-93)

FILING RECEIPT

MAIL ROOM
JUN 28 1994
TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

EH

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTORNEY DOCKET NO.	DRWGS	TOT CL	IND CL
08/146,206	11/17/93	1804	$1,592.00	709P1	9	18	9

JANET E. HASAK
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO, CA 94080-4990

RECEIVED
MAY 13 1994
GENENTECH, INC. LEGAL DEPT.

Receipt is acknowledged of this patent application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please write to the Application Processing Division's Customer Correction Branch within 10 days of receipt. Please provide a copy of the Filing Receipt with the changes noted thereon.

Applicant(s) PAUL J. CARTER, SAN FRANCISCO, CA; LEONARD G. PRESTA, SAN FRANCISCO, CA.

CONTINUING DATA AS CLAIMED BY APPLICANT-
THIS APPLN IS A 371 OF /US92/05126 06/15/92

FOREIGN/PCT APPLICATIONS-PCT PCT/US92/05126 06/15/92

TITLE
IMMUNOGLOBULIN VARIANTS

PRELIMINARY CLASS: 435

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{5} : **C12N 15/13, C12P 21/08** **C07K 13/00, C12N 5/10** **G06F 15/00**	**A1**	**(11) International Publication Number:** **WO 92/22653** **(43) International Publication Date:** 23 December 1992 (23.12.92)

(21) International Application Number: PCT/US92/05126

(22) International Filing Date: 15 June 1992 (15.06.92)

(30) Priority data:
715,272 14 June 1991 (14.06.91) US

(60) Parent Application or Grant
(63) Related by Continuation
US 715,272 (CIP)
Filed on 14 June 1991 (14.06.91)

(71) Applicant *(for all designated States except US):* GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : CARTER, Paul, J. [GB/US]; 2074 18th Avenue, San Francisco, CA 94116 (US). PRESTA, Leonard, G. [US/US]; 1900 Gough Street, #206, San Francisco, CA 94109 (US).

(74) Agents: ADLER, Carolyn, R. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).

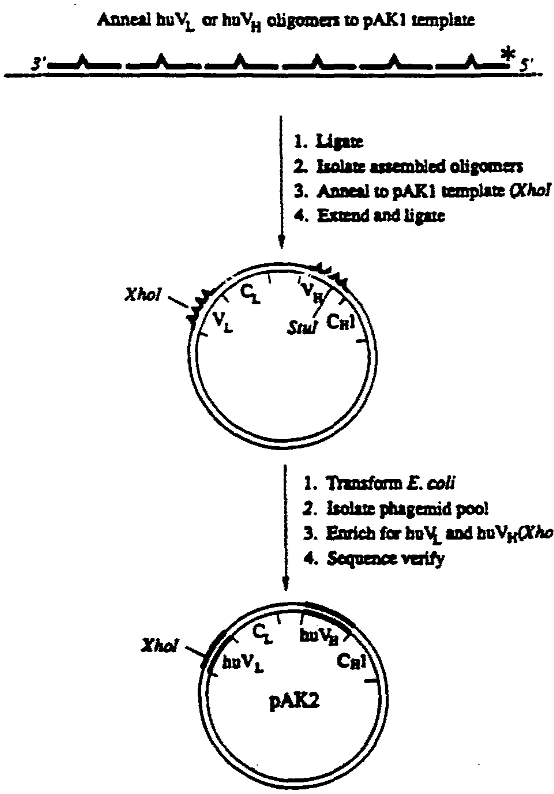
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR MAKING HUMANIZED ANTIBODIES

(57) Abstract

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



08/146,206

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKETT NO.

EXAMINER

ART UNIT	PAPER NUMBER
	53

DATE MAILED: 8/15/94

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) Julie Burke (PTO)
(3) Wendy Lee
(2) Lila Feisee (PTO)
(4)

Date of interview 23 Aug 99

Type: ☐ Telephonic ☒ Personal (copy is given to ☐ applicant ☒ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☐ No. If yes, brief description: None

Agreement ☐ was reached with respect to some or all of the claims in question. ☒ was not reached.

Claims discussed: All pending

Identification of prior art discussed: Reichmann

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: Applicants intend to file TD for claim 111. Applicants intend to add an upper limit to affinity in claim 113 + 128.

(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.

Julie C Burke
Examiner's Signature

PTOL-413 (REV. 2-93)

ORIGINAL FOR INSERTION IN RIGHT HAND FLAP OF FILE WRAPPER

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address : COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/146,206	11/17/93	CARTER	P 709P1

EXAMINER
ADAMS,D

18M2/0826

JANET E. HASAK
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO, CA 94080-4990

ART UNIT	PAPER NUMBER
1806	10

DATE MAILED: 08/26/94

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☐ This application has been examined ☐ Responsive to communication filed on ______ ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 0 month(s), 30 days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice re Patent Drawing, PTO-948.
3. ☐ Notice of Art Cited by Applicant, PTO-1449.
4. ☐ Notice of Informal Patent Application, Form PTO-152.
5. ☐ Information on How to Effect Drawing Changes, PTO-1474.
6. ☐ ______

Part II SUMMARY OF ACTION

1. ☒ Claims 1-18 are pending in the application.

   Of the above, claims ______ are withdrawn from consideration.

2. ☐ Claims ______ have been cancelled.
3. ☐ Claims ______ are allowed.
4. ☐ Claims ______ are rejected.
5. ☐ Claims ______ are objected to.
6. ☒ Claims 1-18 are subject to restriction or election requirement.
7. ☐ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.
8. ☐ Formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on ______. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable. ☐ not acceptable (see explanation or Notice re Patent Drawing, PTO-948).
10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on ______ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed on ______, has been ☐ approved. ☐ disapproved (see explanation).
12. ☐ Acknowledgment is made of the claim for priority under U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. ______ ; filed on ______
13. ☐ Since this application appears to be 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.
14. ☐ Other

PTOL-326 (Rev. 9-89)

EXAMINER'S ACTION

15. Restriction to one of the following inventions is required under 35 U.S.C. § 121:

- I. Claims 1-12,15 and 19, drawn to a method of making a humanized antibody, classified in Class 435, subclasses 69.6, 69.7, 70.21, 91, 172.2, 240.1, 240.27, 252.3, 320.1 and Class 536, subclass 23.53
- II. Claim 13, drawn to a polypeptide, classified in Class 530, subclass 325.
- III. Claim 14, drawn to a polypeptide, classified in Class 530, subclass 325.
- IV. Claim 16, drawn to a computer, classified in Class 364, subclass 413.
- V. Claim 17, drawn to a computer representation, classified in Class 36, subclass 223.3, 223.4, 224.1, 224.91, 225.9 and 226.1
- VI. Claim 18, drawn to a method of storing a computer representation, classified in Class 369, subclass 13+

16. The inventions are distinct, each from the other because of the following reasons:

17. The inventions of Groups I-III are not related. The method of making a humanized antibody of Group I is distinct from the polypeptides of either Groups II or III. The polypeptides are not humanized antibodies. Thus the method of Group I is not expected to produce the polypeptides of Groups II or III. The Groups therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

18. The inventions of Group I and Group VI are distinct methods. They differ with respect to ingredients and method steps. They have different issues regarding patentability and enablement and represent patentably distinct subject matter.

19. The products of Groups II-V are distinct and unrelated. The peptides of Groups II and III differ chemically and physically from a computer and computer representation. Additionally, the peptides have different sequences and thus differed structures and pharmacokinetic properties. The Groups therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

20. The method of Group I is distinct from the products of Groups [IV and V]. The method of Group I can in no manner produce a computer or computer representation as claimed in Groups IV and V. The Groups therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

21. The products of Groups II and III can not be produced by the method of Group VI. They therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

22. The computer of Group IV is distinct from both a method of storing a computer representation of Group VI and a computer representation Group V. The program required for (1) storing or (2) providing a representation (i.e. word processing text) are distinct components from the architecture of a computer system. Thus the Groups are separate and patentably distinct from each other. They have different issues regarding patentability and enablement.

23. The computer representation of Group V is distinct from a method of storing a computer representation. The logic required for these two applications are distinct and unrelated. The Groups have different issues regarding patentability and enablement and represent patentably distinct subject matter.

24. Because these inventions are distinct for the reasons given above and have acquired a separate status in the art shown by their different classification, in addition to their recognized divergent subject matter, they represent an undue burden on the examiner and restriction for examination purposes as indicated is proper.

25. Applicant is advised that the response to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed.

26. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 C.F.R. § 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a diligently-filed petition under 37 C.F.R. § 1.48(b) and by the fee required under 37 C.F.R. § 1.17(h).

27. A telephone call was made to Ms. Hasak on August 24, 1994 to request an oral election to the above restriction requirement, but did not result in an election being made.

28. Papers related to this application may be submitted to Group 180 by facsimile transmission. Papers should be faxed to Group 180 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center telephone number is (703) 308-4227.

29. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Donald E. Adams whose telephone number is (703) 308-0570. The examiner can normally be reached Monday through Thursday from 7:30 am to 6:00 pm. A message may be left on the examiners voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Mr. David Lacey can be reached on (703) 308-3535. The fax phone number for Group 180 is (703) 305-3014 or (703) 308-4227. Any inquiry of a general nature or relating to the status of this application should be directed to the Group 180 receptionist whose telephone number is (703) 308-0196.

August 25, 1994

Donald E. Adams, Ph.D.
Patent Examiner
Group 1800

PATENT DOCKET 709P1 #11

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: 1806
Examiner: D. Adams

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on
September 22, 1994
(Date of Deposit)
Elisa R. Hamby
Name of Depositing Party
Signature of Depositing Party
9/22/94
Date of Signature

TRANSMITTAL LETTER

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Transmitted herewith is a Response to Restriction Requirement 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 Fee(s)
Total	20	Minus	23	= 0	x 22 =	$ 0
Indep.	7	Minus	10	= 0	x 74 =	$ 0
___ First Presentation of Multiple Dependent Claim					+ 230 =	$ 0
					TOTAL	$ 0

_X_ No additional fee is required.
____ The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $. **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.**

A copy of a document pursuant to 37 C.F.R. § 10.9(b) is attached as proof of the authorization of the undersigned to prosecute the above-mentioned application. The original of this document is on file in the Office of Enrollment and Discipline.

Respectfully submitted,
GENENTECH, INC.

Date: September 22, 1994

By: ______________________
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

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 assignee 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 December 9, 1994: (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) Wendy M. Lee ceases to remain or reside in the United States on an 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, 1994

Cameron Weiffenbach
Cameron Weiffenbach, Director
Office of Enrollment and Discipline

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PATENT DOCKET 709P1

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SEP 30 1994
GROUP 1800

1/C
10-4-94

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: 1806
Examiner: D. Adams

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on
September 22, 1994
(Date of Deposit)
Elisa R. Hamby
Name of Depositing Party
Elisa R Hamby
Signature of Depositing Party
9/22/94
Date of Signature

RESPONSE TO RESTRICTION REQUIREMENT

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

This is responsive to the restriction requirement mailed 8/26/94. The period for response has been set for 30 days making this response due on or before 9/25/94. This response is timely filed. Please amend the application as follows:

IN THE CLAIMS:

Please cancel claims 16-18 without prejudice.

REMARKS

The Examiner required restriction to one of the following inventions under 35 USC §121:

I. Claims 1-12 and 15, drawn to a method of making a humanized antibody.
II. Claim 13 drawn to a polypeptide.
III. Claim 14 drawn to a polypeptide.
IV. Claim 16 drawn to a computer.
V. Claim 17 drawn to a computer representation.
VI. Claim 18 drawn to a method of storing a computer representation.

The Examiner urges that the inventions of Groups I-III are not related insofar as the polypeptides of either Groups II or III are "not humanized antibodies" and are therefore distinct from the method of making a humanized antibody recited in claim 1. The Examiner has also taken the position that the method of Group I is not expected to produce the polypeptides of Groups II or III.

Applicants hereby elect Group I, with traverse. The restriction requirement is submitted to be improper as regards the separate treatment of Groups I, II, and III. The claims in the remaining Groups IV, V, and VI have been canceled from this application, without prejudice to file a continuing application directed thereto.

It is submitted that the inventions of Groups I, II, and III as hereinabove defined are not distinct. These inventions are all respectively related as method of making a humanized antibody (Group I) and the humanized antibody made using the method of claim 1. Applicants submit that the assumption made that the polypeptides of claims 13 and 14 are not humanized antibodies is clearly in error. In particular, claims 13 and 14 encompass the light chain and heavy chain variable domain, respectively, of humanized MAb4D5 made using the method of claim 1 (see page 7, lines 13-21 and Example 1 which describes humanization of muMAb4D5). Surely, the Examiner will agree that the claim encompassing the light chain variable domain of the humanized MAb4D5 (claim 13) and the claim to the heavy chain variable domain of this humanized antibody (claim 14) should be examined together, since both a heavy chain and a light chain are required to form the antibody variable domain. Hence, the separate treatment of Groups II and III is clearly erroneous. Furthermore, since the humanized antibody variable domains of claims 13 and 14 are made using the humanization technique of claim 1, these claims should be examined together.

With respect to the search required to determine the patentability of the inventions defined by the claims of Groups I, II, and III, applicants represent that it is impossible to conduct an exhaustive search for a method of making a humanized antibody without searching for humanized antibodies made using the method. Similarly, the search for the claimed humanized antibody is bound to reveal information concerning the technique for humanizing it. In the same token, a search of the amino acid sequence encoding the humanized heavy chain variable domain of the antibody would lead to the discovery of information concerning the humanized light chain variable domain. Accordingly, performing the entire search covering the method and products made by the method is less burdensome on the Examiner than the separate search, which necessarily involves duplication of searching efforts.

In view of the foregoing arguments, the Examiner is requested to reconsider and withdraw the restriction requirement.

A copy of a document pursuant to 37 C.F.R. § 10.9(b) is attached as proof of the authorization of the undersigned to prosecute the above-mentioned application. The original of this document is on file in the Office of Enrollment and Discipline.

Respectfully submitted,

GENENTECH, INC.

Date: Sept 22, 1994

By: W M Lee

Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

```
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
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Ser Gly Leu Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
                110                 115                 120

Ser Ser
    122
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(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:

F1

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Asn Gln Arg Phe Met Asp Lys Ala Thr Leu Ala Val Asp Lys Ser
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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
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F1

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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
```

F1

```
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
```

f1

(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
```

R1

(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
```

F1

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Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val
                200                 205                 210

Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr
                215                 220                 225

Lys Ser Phe Asn Arg Gly Glu Cys
                230         233
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1                5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
                 20                  25                  30

Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45
```

```
Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Thr Thr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
                65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                95                  100                 105

Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
                110                 115                 120

Ser Ser
    122
```

F1

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Patent and Trademark Office
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Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/146.206	11/17/93	CARTER	P-709P1

EXAMINER
ADAMS,D

18M2/1209

JANET E. HASAK
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
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ART UNIT	PAPER NUMBER
1806	12

DATE MAILED:
12/09/94

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

Election, Amdt C

☒ This application has been examined ☒ Responsive to communication filed on 9/26/94 ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), 0 days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☒ Notice of References Cited by Examiner, PTO-892.
2. ☒ Notice re Patent Drawing, PTO-948.
3. ☒ Notice of Art Cited by Applicant, PTO-1449.
4. ☐ Notice of Informal Patent Application, Form PTO-152.
5. ☒ Information on How to Effect Drawing Changes, PTO-1474.
6. ☐ ____

Part II SUMMARY OF ACTION

1. ☒ Claims 1-15 are pending in the application.

   Of the above, claims 13 & 14 are withdrawn from consideration.

2. ☒ Claims 16-18 have been cancelled.
3. ☐ Claims ____ are allowed.
4. ☒ Claims 1-12 & 15 are rejected.
5. ☐ Claims ____ are objected to.
6. ☐ Claims ____ are subject to restriction or election requirement.
7. ☒ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.
8. ☐ Formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on ____. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable. ☐ not acceptable (see explanation or Notice re Patent Drawing, PTO-948).
10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on ____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed on ____, has been ☐ approved. ☐ disapproved (see explanation).
12. ☐ Acknowledgment is made of the claim for priority under U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. ____ ; filed on ____.
13. ☐ Since this application appears to be 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.
14. ☐ Other

EXAMINER'S ACTION

PTOL-326 (Rev. 9-89)

15. Applicant's election with traverse of Group I, claims 1-12 and 15 in Paper No. 11 is acknowledged. The traversal is on the ground(s) that:
(1) the inventions are all respectively related as method of making a humanized antibody. Contrary to applicant's belief the polypeptides of claim 13 (admittedly drawn to the light chain of humanized MAb4D5) and claim 14 (admittedly drawn to the heavy chain of humanized Mab4D5) are not methods.

(2) the assumption made that the polypeptides of claims 13 and 14 are not humanized antibodies is clearly in error. Applicant is invited to reconsider this position since the antibodies of Group I are composed of a heavy and light chains. A polypeptide of just the light chain (claims 13) or just the heavy chain (claim 14) is not an antibody as prepared by Group I. The Groups therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

(3) applicants represent that it is impossible to conduct an exhaustive search for a method of making a humanized antibody without searching for humanized antibodies made using the method. To demonstrate the problem with this argument applicant is invited to consider the classification of the Groups found in the restriction requirement. Note that the Groups are classified into distinct classifications. Thus, an exhaustive search would clearly not require searching for polypeptides. Additionally, the light chain (claim 13) and heavy chain (claim 14) are distinct from the antibodies of Group I. Again, a search of Group I would not require the search of a polypeptide.

As a whole applicant's arguments were not found persuasive.
The requirement is still deemed proper and is therefore made FINAL.

16. Claims 16-18 have been cancelled.

17. Claims 13 and 14 have been withdrawn as directed to a non-elected invention.

18. Claims 1-12 and 15 are currently under consideration.

19. The oath or declaration is defective. A new oath or declaration in compliance with 37 C.F.R. § 1.67(a) identifying this application by its Serial Number and filing date is required. See M.P.E.P. §§ 602.01 and 602.02.

20. The oath or declaration is defective because:
It does not state that the person making the oath or declaration in a continuation-in-part application filed under the conditions specified in 35 U.S.C. § 120 which discloses and

claims subject matter in addition to that disclosed in the prior copending application, acknowledges the duty to disclose material information as defined in 37 C.F.R. § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

21. This application has been filed with informal drawings which are acceptable for examination purposes only. Formal drawings will be required when the application is allowed.

22. Applicant is required to submit a proposed drawing correction in response to this Office action. However, correction of the noted defect can be deferred until the application is allowed by the examiner.

23. The following is a quotation of the first paragraph of 35 U.S.C. § 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

24. The specification is objected to under 35 U.S.C. § 112, first paragraph, as failing to provide an adequate written description of the invention and for failing to adequately teach how to make and/or use the invention, i.e. for failing to provide an enabling disclosure.

A) Applicants have not disclosed to one of any skill in the art how to use the claimed antibody or antibody produced by the claimed methods. The scope of the claims reads on any antibody. It is unclear from the specification if the methods or antibodies claimed will all have a diagnostic or therapeutic utility. Applicant has exemplified only one such antibody specifically MAb4D5, as having diagnostic utility for the detection of $p185^{HER2}$. It is unclear if any other antibody will have a diagnostic or therapeutic utility. Determining which other antibodies are useful would be an unpredictable event and would require undue experimentation for a person of any skill in the art to get from what the specification has disclosed to the claimed invention.

25. Claims 1-12 and 15 are rejected under 35 U.S.C. § 112, first paragraph, for the reasons set forth in the objection to the specification.

26. Claims 1, 2, 4-12 and 15 are rejected under 35 U.S.C. § 103 as being unpatentable over Winter [EP 0239400], Riechmann et al. [Nature 332:323-327 (1988)] and Queen et al. [Proc. Natl. Acad. Sci. 86:10029-10033 (1989)]. Briefly the claims are drawn to a method for producing humanized antibodies and humanized antibodies. Winter, teaches the production of altered, chimeric, antibodies by replacing the complementarily determining regions (CDRs), see abstract. Winter, teaches the requirements for CDR fusions, see page 6 to page 8, line 29. Particularly, page 8, lines 11-18, where Winter, teaches that "merely by replacing one or more CDRs with complementary CDRs may not always result in a functional altered antibody..... it will be well within the competence of the man skilled in the art, either by carrying out routine experimentation or by trail and error testing to obtain a functional altered antibody. Note at page 8, last full paragraph that Winter states that framework region replacement and sequence changing may be necessary to obtain a functional humanized antibody. On page 9, lines 13-16, Winter suggests that the antibodies would be of importance for use in human therapy. Winter, teaches a method of producing the antibody, see page 10, paragraph 3 to page 15, paragraph 2. Consistent with Winter, Riechmann et al. teach a method of reshaping human antibodies for therapy by CDR grafting, see whole document and Queen et al. teach the humanization of antibodies by CDR grafting, see entire document. Riechmann et al. teach altering the sequence of the antibody to restore packing or to increase binding affinity, see page 326, first column, first full paragraph. Queen et al. teach the use of computer modeling to assist in the production of humanized antibodies, specifically to predict which amino acids to change thereby effecting molecular interactions, note that of the amino acids predicted to change include those identified by applicant in claims 7 and 10. A person of ordinary skill in the art would have realized that dependent upon the framework region selected and the sequence of the CDR regions amino acid changes would need to be made and they would depend upon the precise amino acid interactions of the polypeptide. The combination of Winter, Riechmann et al. and Queen et al. teach a comprehensive method for producing humanized antibodies which include the steps outlined in applicant's claims. Therefore, it would have been <u>prima facia</u> obvious to a person of ordinary skill in the art at the time the invention was made to take the combined teachings of Winter, Riechmann et al. and Queen et al. to produce a method of making a humanized antibody and to have a humanized antibody for either diagnostic or therapeutic use.

27. Claims 1, 2, 4-12 and 15 are rejected under 35 U.S.C. § 103 as being unpatentable over Winter [EP 0239400], Riechmann et al. [Nature 332:323-327 (1988)] and Queen et al. [Proc. Natl. Acad. Sci. 86:10029-10033 (1989)] in view of <u>In re Durden</u> 226 U.S.P.Q. 359 (Fed. Cir. 1985). Briefly the claims are drawn to a method

for producing humanized antibodies and humanized antibodies. As discussed above the combination of Winter, Riechmann et al. and Queen et al. teach humanized antibodies and methods for their production. Applicant's claimed invention does not appear to differ from what has previously known in the art.

28. Claim 3 is rejected under 35 U.S.C. § 103 as being unpatentable over Winter [EP 0239400], Riechmann et al. [Nature 332:323-327 (1988)] and Queen et al. [Proc. Natl. Acad. Sci. 86:10029-10033 (1989)] as applied to claims 1, 2, 4-12 and 15 and further in view of Roitt [Immunology, published 1985, by Gower Medical Publishing Ltd. (London, England) page 5.5]. Briefly the claim is drawn to a method for producing humanized antibodies having the additional steps of searching the import variable domain sequence for glycosylation sites, determining if any such glycosylation site is reasonable expected to affect the antigen binding or affinity of the antibody and if so substituting the glycosylation site into the consensus sequence. As discussed above the combination of Winter, Riechmann et al. and Queen et al. teach humanized antibodies and methods of producing humanized antibodies. The combination of Winter, Riechmann et al. and Queen et al. do not teach the importance of carbohydrate residues. However, Roitt teaches that antibodies contain carbohydrate residues in the variable region. A person of ordinary skill in the art would realize that carbohydrate residues can produce steric modifications in the folding characteristics of polypeptides. Therefore it would have been prima facia obvious to a person of ordinary skill in the art at the time the invention was made to include a step in the method taught by the combination of Winter, Riechmann et al. and Queen et al. which determines if the presence of carbohydrate residues occur in the variable region that can affect antigen binding and then include in the antibody sequence the appropriate glycosylation signal, by adding the appropriate consensus sequence. A person of ordinary skill in the art would have been motivated to add the additional step of identifying glycosylation that may affect antigen binding to ensure that the antibody produced will have the appropriate binding affinity. A person of ordinary skill in the art would have been motivated to produce such an method to produce antibodies having diagnostic or therapeutic utility.

29. Applicant is invited to include continuing data at the first page of the specifiation which identifies all related applications and noting their current status.

30. No claim allowed.

31. Papers related to this application may be submitted to Group 180 by facsimile transmission. Papers should be faxed to Group

180 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center telephone number is (703) 308-4227.

32. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Donald E. Adams whose telephone number is (703) 308-0570. The examiner can normally be reached Monday through Thursday from 7:30 am to 6:00 pm. A message may be left on the examiners voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Mr. David Lacey can be reached on (703) 308-3535. The fax phone number for Group 180 is (703) 305-3014 or (703) 308-4227. Any inquiry of a general nature or relating to the status of this application should be directed to the Group 180 receptionist whose telephone number is (703) 308-0196.

December 5, 1994

Donald E. Adams, Ph.D.
Patent Examiner
Group 1800

PTO 892 DEA/FCE 1994 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE NOTICE OF REFERENCES CITED	SERIAL NUMBER 08/146,206	Art Unit 1806	Attachment to Paper Number 12
	APPLICANT(S) : Carter et al.		

U.S. PATENT DOCUMENTS

*		DOCUMENT NUMBER	DATE	NAME(S)	CLASS	SUBCLASS	FILING DATE

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS	PERTINENT DRW	PERTINENT SPEC
	A	0 239 400	09/30/87	EP	Winter	C12N	15/00	-	-
*	B	90/07861	26/07/90	WO	Queen	C12P	21/00	-	-

*		OTHER REFERENCES (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)
	C	Riechmann et al. [Nature 332:323-327 (1988)]
	D	Queen et al. [Proc. Natl. Acad. Sci. 86:10029-10033 (1989)]
	E	Roitt [Immunology, published 1985, by Gower Medical Publishing Ltd. (London, England) page 5.5]
*	F	Tramontano et al. [J. Mol. Biol. 215:175-182 (1990)]

EXAMINER	DATE	* A COPY OF THIS REFERENCE IS NOT BEING FURNISHED WITH THIS OFFICE ACTION. (SEE MPEP SECTION 707.05(a).
	12/5/94	PAGE 1 OF 1

TDias 12/05/01

⑲ Europäisches Patentamt
European Patent Office
Office européen des brevets

⑪ Publication number: **0 239 400**
A2

⑫

EUROPEAN PATENT APPLICATION

㉑ Application number: **87302620.7**

㉒ Date of filing: **26.03.87**

(51) Int. Cl.⁴: **C 12 N 15/00**, C 07 K 15/06, C 12 P 21/02

㉚ Priority: **27.03.86 GB 8607679**

㊸ Date of publication of application: **30.09.87**
Bulletin 87/40

(84) Designated Contracting States: **AT BE CH DE ES FR GB GR IT LI LU NL SE**

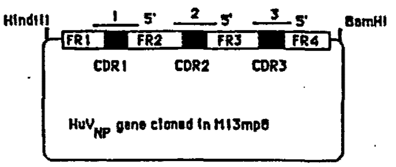
(71) Applicant: **Winter, Gregory Paul, 64 Cavendish Avenue, Cambridge (GB)**

(72) Inventor: **Winter, Gregory Paul, 64 Cavendish Avenue, Cambridge (GB)**

(74) Representative: **Votier, Sidney David et al, CARPMAELS & RANSFORD 43, Bloomsbury Square, London WC1A 2RA (GB)**

(54) **Recombinant antibodies and methods for their production.**

(57) An altered antibody is produced by replacing the complementarity determining regions (CDRs) of a variable region of an immunoglobulin (Ig) with the CDRs from an Ig of different specificity, using recombinant DNA techniques. The gene coding sequences for producing the altered antibody may be produced by site-directed mutagenesis using long oligonucleotides.



D1.3 CDR1 oligonucleotide
5' CTG,TCT,CAC,CCA,GTT,TAC,ACC,ATA,GCC,GCT,GAA,GGT,GCT
FR2 D1.3 CDR1 FR1

D1.3 CDR2 oligonucleotide
5' CAT,TGT,CAC,TCT,GGA,TTT,GAG,AGC,TGA,ATT,ATA,GTC,TGT,
FR3 D1.3 CDR2
GTT,TCC,ATC,ACC,CCA,AAT,CAT,TCC,AAT,CCA,CTC
D1.3 CDR2 FR2

D1.3 CDR3 oligonucleotide
5' GCC,TTG,ACC,CCA,GTA,GTC,AAG,CCT,ATA,ATC,TCT,CTC,TCT,
FR4 D1.3 CDR3
TGC,ACA,ATA
FR3

EP 0 239 400 A2

ACTORUM AG

TITLE MODIFIED
see front page

RECOMBINANT DNA PRODUCT AND METHODS

The present invention relates to altered antibodies in which at least parts of the complementarity determining regions (CDRs) in the light or heavy chain variable domains of the antibody have been replaced by analogous parts of CDRs from an antibody of different specificity. The present invention also relates to methods for the production of such altered antibodies.

Natural antibodies, or immunoglobulins, comprise two heavy chains linked together by disulphide bonds and two light chains, one light chain being linked to each of the heavy chains by disulphide bonds. The general structure of an antibody of class IgG (i.e. an immunoglobulin (Ig) of class gamma (G)) is shown schematically in Figure 1 of the accompanying drawings.

Each heavy chain has at one end a variable domain followed by a number of constant domains. Each light chain has a variable domain at one end and a constant domain at its other end, the variable domain being aligned with the variable domain of the heavy chain and the constant domain being aligned with the first constant domain of the heavy chain. The constant domains in the light and heavy chains are not involved directly in binding the antibody to the antigen.

The variable domains of each pair of light and heavy chains form the antigen binding site. The domains on the light and heavy chains have the same general structure and each domain comprises four framework regions, whose sequences are relatively conserved, connected by three hypervariable or complementarity determining regions (CDRs) (see Kabat, E.A., Wu, T.T., Bilofsky, H., Reid-Miller, M. and Perry, H., in "Sequences of Proteins of Immunological Interest", US Dept. Health and Human Services 1983). The four framework regions largely adopt a β-sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs are held in close proximity by the framework regions and, with the CDRs from the other domain, contribute to the formation of the antigen binding site.

For a more detailed account of the structure of variable domains, reference may be made to: Poljak, R.J., Amzel, L.M., Avey, H.P., Chen, B.L., Phizackerly, R.P. and Saul, F., PNAS USA, 70, 3305-3310, 1973; Segal, D.M., Padlan, E.A., Cohen, G.H., Rudikoff, S., Potter, M. and Davies, D.R., PNAS USA, 71, 4298-4302, 1974; and Marquart, M., Deisenhofer, J., Huber, R. and Palm, W., J. Mol. Biol., 141, 369-391, 1980.

In recent years advances in molecular biology based on recombinant DNA techniques have provided processes for the production of a wide range of heterologous polypeptides by transformation of host cells with heterologous DNA sequences which code for the production of the desired products.

EP-A-0 088 994 (Schering Corporation) proposes the construction of recombinant DNA vectors comprising a ds DNA sequence which codes for a variable domain of a light or a heavy chain of an Ig specific for a predetermined ligand. The ds DNA sequence is provided with initiation and termination codons at its 5'- and 3'- termini respectively, but lacks any nucleotides coding for amino acids superfluous to the variable domain. The ds DNA sequence is used to transform bacterial cells. The application does not contemplate variations in the sequence of the variable domain.

EP-A-1 102 634 (Takeda Chemical Industries Limited) describes the cloning and expression in bacterial host organisms of genes coding for the whole or a part of human IgE heavy chain polypeptide, but does not contemplate variations in the sequence of the polypeptide.

EP-A-0 125 023 (Genentech Inc.) proposes the use of recombinant DNA techniques in bacterial cells to produce Ig's which are analogous to those normally found in vertebrate systems and to take advantage of the gene modification techniques proposed therein to construct chimeric Igs or other modified forms of Ig.

The term 'chimeric antibody' is used to describe a protein comprising at least the antigen binding portion of an immunoglobulin molecule (Ig) attached by peptide linkage to at least part of another protein.

It is believed that the proposals set out in the above Genentech application did not lead to the expression of any significant quantities of Ig polypeptide chains, nor to the production of Ig activity, nor to the secretion and assembly of the chains into the desired chimeric Igs.

The production of monoclonal antibodies was first disclosed by Kohler and Milstein (Kohler, G. and Milstein, C., Nature, 256, 495-497, 1975). Such monoclonal antibodies have found widespread use not only as diagnostic reagents (see, for example, 'Immunology for the 80s, Eds. Voller, A., Bartlett, A., and Bidwell, D., MTP Press, Lancaster, 1981) but also in therapy (see, for example, Ritz, J. and Schlossman, S.F., Blood, 59, 1-11, 1982).

The recent emergence of techniques allowing the stable introduction of Ig gene DNA into myeloma cells (see, for example, Oi, V.T., Morrison, S.L., Herzenberg, L.A. and Berg, P., PNAS USA, 80, 825-829, 1983; Neuberger, M.S., EMBO J., 2, 1373-1378, 1983; and Ochi, T., Hawley, R.G., Hawley, T., Schulman, M.J., Traunecker, A., Kohler, G. and Hozumi, N., PNAS USA, 80, 6351-6355, 1983), has opened up the possibility of using *in vitro* mutagenesis and DNA transfection to construct recombinant Igs possessing novel properties.

However, it is known that the function of an Ig molecule is dependent on its three dimensional structure, which in turn is dependent on its primary amino acid sequence. Thus, changing the amino acid sequence of an Ig may adversely affect its activity. Moreover, a change in the DNA sequence coding for the Ig may affect the ability of the cell containing the DNA sequence to express, secrete or assemble the Ig.

It is therefore not at all clear that it will be possible to produce functional altered antibodies by recombinant DNA techniques.

However, colleagues of the present Inventor have devised a process whereby chimeric antibodies in which both parts of the protein are functional can be secreted. The process, which is disclosed in International Patent Application No. PCT/GB85/00392 (Neuberger et al. and Celltech Limited), comprises:

a) preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence comprising a first part which encodes at least the variable domain of the heavy or light chain of an Ig molecule and a second part which encodes at least part of a second protein;

b) if necessary, preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary light or heavy chain respectively of an Ig molecule;

c) transforming an immortalised mammalian cell line with the or both prepared vectors; and

d) culturing said transformed cell line to produce a chimeric antibody.

The second part of the DNA sequence may encode:

i) at least part, for instance the constant domain of a heavy chain, of an Ig molecule of different species, class or subclass;

ii) at least the active portion or all of an enzyme;

iii) a protein having a known binding specificity;

iv) a protein expressed by a known gene but whose sequence, function or antigenicity is not known; or

v) a protein toxin, such as ricin.

The above Neuberger application only shows the production of chimeric antibodies in which complete variable domains are coded for by the first part of the DNA sequence. It does not show any chimeric antibodies in which the sequence of the variable domain has been altered.

The present invention, in a first aspect, provides an altered antibody in which at least parts of the CDRs in the light or heavy chain variable domains have been replaced by analogous parts of CDRs from an antibody of different specificity

The determination as to what constitutes a CDR and what constitutes a framework region was made on the basis of the amino-acid sequences of a number of Igs. However, from the three dimensional structure of a number of Igs it is apparent that the antigen binding site of an Ig variable domain comprises three looped regions supported on sheet-like structures. The loop regions do not correspond exactly to the CDRs, although in general there is considerable overlap.

Moreover, not all of the amino-acid residues in the loop regions are solvent accessible and in one case, amino-acid residues in the framework regions are involved in antigen binding.(Amit, A.G., Mariuzza, R.A., Phillips, S.E.V. and Poljak, R.J., Science, <u>233</u>, 747-753, 1986).

It is also known that the variable regions of the two parts of an antigen binding site are held in the correct orientation by inter-chain non-covalent interactions. These may involve amino-acid residues within the CDRs.

Thus, in order to transfer the antigen binding capacity of one variable domain to another, it may not be necessary to replace all of the CDRs with the complete CDRs from the donor variable region. It may be necessary only to transfer those residues which are accessible from the antigen binding site, and this may involve transferring framework region residues as well as CDR residues.

It may also be necessary to ensure that residues essential for inter-chain interactions are preserved in the acceptor variable domain.

Within a domain, the packing together and orientation of the two disulphide bonded β-sheets (and therefore the ends of the CDR loops) are relatively conserved. However, small shifts in packing and orientation of these β-sheets do occur

(Lesk, A.M. and Chothia, C., J. Mol. Biol., 160, 325-342, 1982). However, the packing together and orientation of heavy and light chain variable domains is relatively conserved (Chothia, C., Novotny, J., Bruccoleri, R. and Karplus, M., J. Mol. Biol., 186, 651-653, 1985). These points will need to be borne in mind when constructing a new antigen biding site so as to ensure that packing and orientation are not altered to the deteriment of antigen binding capacity.

It is thus clear that merely by replacing one or more CDRs with complementary CDRs may not always result in a functional altered antibody. However, given the explanations set out above, it will be well within the competence of the man skilled in the art, either by carrying out routine experimentation or by trial and error testing to obtain a functional altered antibody.

Preferably, the variable domains in both the heavy and light chains have been altered by at least partial CDR replacement and, if necessary, by partial framework region replacement and sequence changing. Although the CDRs may be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of different class and preferably from an antibody from a different species.

Thus, it is envisaged, for instance, that the CDRs from a mouse antibody could be grafted onto the framework regions of a human antibody. This arrangement will be of particular use in the therapeutic use of monoclonal antibodies.

At present, when a mouse monoclonal antibody or even a chimeric antibody comprising a complete mouse variable domain is injected into a human, the human body's immune system recognises the mouse variable domain as foreign and produces an immune response thereto. Thus, on subsequent injections of the mouse antibody or chimeric antibody into the human, its effectiveness is considerably reduced by the action of the body's immune system against the foreign antibody. In the altered antibody of the present invention, only the CDRs of the antibody will be foreign to the body, and this should minimise side effects if used for human therapy. Although, for example, human and mouse framework regions have characteristic sequences, there seem to be no characteristic features which distinguish human from mouse CDRs. Thus, an antibody comprised of mouse CDRs in a human framework may well be no more foreign to the body than a genuine human antibody.

Even with the altered antibodies of the present invention, there is likely to be an anti-idiotypic response by the recipient of the altered antibody. This response is directed to the antibody binding region of the altered antibody, It is believed that at least some anti-idiotype antibodies are directed at sites bridging the CDRs and the framework regions. It would therefore be possible to provide a panel of antibodies having the same partial or complete CDR replacements but on a series of different framework regions. Thus, once a first altered antibody became therapeutically ineffective, due to an anti-idiotype response, a second altered antibody from the series could be used, and so on, to overcome the effect of the anti-idiotype response. Thus, the useful life of the antigen-binding capacity of the altered antibodies could be extended.

Preferably, the altered antibody has the structure of a natural antibody or a fragment thereof. Thus, the altered antibody may comprise a complete antibody, an $(Fab')_2$ fragment, an Fab fragment, a light chain dimer or a heavy chain dimer. Alternatively, the altered antibody may be a chimeric antibody of the type described in the Neuberger application referred to above. The production of such an altered chimeric antibody can be carried out using the methods described below used in conjunction with the methods described in the Neuberger application.

The present invention, in a second aspect, comprises a method for producing such an altered antibody comprising:

a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least a variable domain of an Ig heavy or light chain, the variable domain comprising framework regions from a first antibody and CDRs comprising at least parts of the CDRs from a second antibody of different specificity;

b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary Ig light or heavy chain respectively;

c) transforming a cell line with the first or both prepared vectors; and

d) culturing said transformed cell line to produce said altered antibody.

The present invention also includes vectors used to transform the cell line, vectors used in producing the transforming vectors, cell lines transformed with the transforming vectors, cell lines tranformed with preparative vectors, and methods for their production.

Preferably, the cell line which is transformed to produce the altered antibody is an immortalised mammalian cell line, which is advantageously of lymphoid origin, such as a myeloma, hybridoma, trioma or quadroma cell line. The cell line may also comprise a normal lymphoid cell, such as a B-cell, which has been immortalised by transformation with a virus, such as the Epstein-Barr virus. Most preferably, the immortalised cell line is a myeloma cell line or a derivative thereof.

Although the cell line used to produce the altered antibody is preferably a mammalian cell line, any other suitable cell line, such as a bacterial cell line or a yeast cell line, may alternatively be used. In particular, it is envisaged that E. Coli derived bacterial strains could be used.

It is known that some immortalised lymphoid cell lines, such as myeloma cell lines, in their normal state secrete isolated Ig light or heavy chains. If such a cell line is transformed with the vector prepared in step a) of the process of the invention, it will not be necessary to carry out step b) of the process, provided that the normally secreted chain is complementary to the variable domain of the Ig chain encoded by the vector prepared in step a).

However, where the immortalised cell line does not secrete or does not secrete a complementary chain, it will be necessary to carry out step b). This step may be carried out by further manipulating the vector produced in step a) so that this vector encodes not only the variable domain of an altered antibody light or heavy chain, but also the complementary variable domain.

Alternatively, step b) is carried out by preparing a second vector which is used to transform the immortalised cell line. This alternative leads to easier construct preparation, but may be less preferred than the first alternative in that it may not lead to as efficient production of antibody.

The techniques by which such vectors can be produced and used to transform the immortalised cell lines are well known in the art, and do not form any part of the invention.

In the case where the immortalised cell line secretes a complementary light or heavy chain, the transformed cell line may be produced for example by transforming a suitable bacterial cell with the vector and then fusing the bacterial cell with the immortalised cell line by spheroplast fusion. Alternatively, the DNA may be directly introduced into the immortalised cell line by electroporation.

The DNA sequence encoding the altered variable domain may be prepared by oligonucleotide synthesis. This requires that at least the framework region sequence of the acceptor antibody and at least the CDRs sequences of the donor antibody are known or can be readily determined. Although determining these sequences, the synthesis of the DNA from oligonucleotides and the preparation of suitable vectors is to some extent laborious, it involves the use of known techniques which can readily be carried out by a person skilled in the art in light of the teaching given here.

If it was desired to repeat this strategy to insert a different antigen binding site, it would only require the synthesis of oligonucleotides encoding the CDRs, as the framework oligonucleotides can be re-used.

A convenient variant of this technique would involve making a symthetic gene lacking the CDRs in which the four framework regions are fused together with suitable restriction sites at the junctions. Double stranded synthetic CDR cassettes with sticky ends could then be ligated at the junctions of the framework regions. A protocol for achieving this variant is shown diagrammatically in Figure 6 of the accompanying drawings.

Alternatively, the DNA sequence encoding the altered variable domain may be prepared by primer directed oligonucleotide site-directed mutagenesis. This technique in essence involves hybridising an oligonucleotide coding for a desired mutation with a single strand of DNA containing the region to be mutated and using the single strand as a template for extension of the oligonulcleotide to produce a strand containing the mutation. This technique, in various forms, is described by : Zoller, M.J. and Smith, M., Nuc. Acids Res., 10, 6487-6500, 1982; Norris, K., Norris F., Christiansen, L. and Fiil, N., Nuc. Acids Res., 11, 5103-5112, 1983; Zoller, M.J. and Smith, M., DNA, 3, 479-488 (1984); Kramer, W., Schughart, K. and Fritz, W.-J., Nuc. Acids Res., 10, 6475-6485, 1982.

For various reasons, this technique in its simplest form does not always produce a high frequency of mutation. An improved technique for introducing both single and multiple mutations in an M13 based vector, has been described by Carter et al. (Carter, P., Bedouelle H. and Winter, G., Nuc. Acids Res., 13, 4431-4443, 1985)

Using a long oligonucleotide, it has proved possible to introduce many changes simultaneously (as in Carter et al., loc. cit.) and thus single oligonucleotides, each encoding a CDR, can be used to introduce the three CDRs from a second antibody into the framework regions of a first antibody. Not only is this technique less laborious than total gene synthesis, but it represents a particularly convenient way of expressing a variable domain of required specificity, as it can be simpler than tailoring an entire V_H domain for insertion into an expression plasmid.

The oligonucleotides used for site-directed mutagenesis may be prepared by oligonucleotide synthesis or may be isolated from DNA coding for the variable domain of the second antibody by use of suitable restriction enzymes. Such long oligonucleotides will generally be at least 30 bases long and may be up to or over 80 bases in length.

The techniques set out above may also be used, where necessary, to produce the vector of part (b) of the process.

The method of the present invention is envisaged as being of particular use in "humanising" non-human monoclonal antibodies. Thus, for instance, a mouse monoclonal antibody against a particular human cancer cell may be produced by techniques well known in the art. The CDRs from the mouse monoclonal antibody may then be partially or totally grafted into the framework regions of a human monoclonal antibody, which is then produced in quantity by a suitable cell line. The product is thus a specifically targetted, essentially human antibody which will recognise the cancer cells, but will not itself be recognised to any significant degree, by a human's immune system, until the anti-idiotype response eventually becomes apparent. Thus, the method and product of the present invention will be of particular use in the clinical environment.

The present invention is now described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 is a schematic diagram showing the structure of an IgG molecule;

Figure 2 shows the amino acid sequence of the V_H domain of NEWM in comparison with the V_H domain of the BI-8 antibody;

Figure 3 shows the amino acid and nucleotide sequence of the HuV_{NP} gene;

Figure 4 shows a comparison of the results for HuV_{NP}-IgE and MoV_{NP}-IgE in binding inhibition assays;

Figure 5 shows the structure of three oligonucleotides used for site directed mutagenesis;

Figure 6 shows a protocol for the construction of CDR replacements by insertion of CDR cassettes into a vector containing four framework regions fused together;

Figure 7 shows the sequence of the variable domain of antibody D1.3 and the gene coding therefor; and

Figure 8 shows a protocol for the cloning of the D1.3 variable domain gene.

EXAMPLE 1

This example shows the production of an altered antibody in which the variable domain of the heavy chains comprises the framework regions of a human heavy chain and the CDRs from a mouse heavy chain.

The framework regions were derived from the human myeloma heavy chain NEWM, the crystallographic structure of which is known (see Poljak et al., loc. cit. and Reth, M., Hammerling, G.J. and Rajewsky, K., EMBO J., 1, 629-634, 1982.)

The CDRs were derived from the mouse monoclonal antibody B1-8 (see Reth et al., loc. cit.), which binds the hapten NP-cap (4-hydroxy-3-nitrophenyl acetyl-caproic acid: K_{NP-CAP}=1.2 μM).

A gene encoding a variable domain HuV_{NP}, comprising the B1-8 CDRs and the NEWM framework regions, was constructed by gene synthesis as follows.

The amino acid sequence of the V_H domain of NEWM is shown in Figure 2, wherein it is compared to the amino acid sequence of the V_H domain of the B1-8 antibody. The sequence is divided into framework regions and CDRs according to Kabat et al. (loc. cit.). Conserved residues are marked with a line.

The amino acid and nucleotide sequence of the HuV_{NP} gene, in which the CDRs from the B1-8 antibody alternate with the framework regions of the NEWM antibody, is shown in Figure 3. The HuV_{NP} gene was derived by replacing sections of the MoV_{NP} gene in the vector pSV-V_{NP} (see Neuberger, M.S., Williams, G.T., Mitchell, E.B., Jouhal, S., Flanagan, J.G. and Rabbitts, T.H., Nature, 314, 268-270, 1985) by a synthetic fragment encoding the HuV_{NP} domain. Thus the 5' and 3' non-coding sequences, the leader sequence, the L-V intron, five N-terminal and four

C-terminal amino acids are from the MoV_{NP} gene and the rest of the coding sequence is from the synthetic HuV_{NP} fragment.

The oligonucleotides from which the HuV_{NP} fragment was assembled are aligned below the corresponding portion of the HuV_{NP} gene. For convenience in cloning, the ends of oligonucleotides 25 and 26b form a Hind II site followed by a Hind III site, and the sequences of the 25/26b oligonucleotides therefore differ from the HuV_{NP} gene.

The HuV_{NP} synthetic fragment was built as a PstI-Hind III fragment. The nucleotide sequence was derived from the protein sequence using the computer programme ANALYSEQ (Staden, R., Nuc. Acids. Res., 12, 521-538, 1984) with optimal codon usage taken from the sequences of mouse constant domain genes. The oligonucleotides (1 to 26b, 28 in total) vary in size from 14 to 59 residues and were made on a Biosearch SAM or an Applied Biosystems machine, and purified on 8M-urea polyacrylamide gels (see Sanger, F. and Coulson, A., FEBS Lett., 87, 107-110, 1978).

The oligonucleotides were assembled in eight single stranded blocks (A-D) containing oligonucleotides

[1,3,5,7] (Block A), [2,4,6,8] (block A'), [9,11,13a,13b] (Block B), [10a, 10b,12/14] (block B'), [15, 17] (block C), [16,18] (block C'), [19, 21, 23, 25] (block D) and [20, 22/24, 26a, 26b) (block D').

In a typical assembly, for example of block A, 50 pmole of oligonucleotides 1,3,5 and 7 were phosphorylated at the 5' end with T4 polynucleotide kinase and mixed together with 5 pmole of the terminal oligonucleotide [1] which had been phosphorylated with 5 μCi [γ-^{32}P] ATP (Amersham 3000 Ci/mmole). These oligonucleotides were annealed by heating to 80°C and cooling over 30 minutes to room temperature, with unkinased oligonucleotides 2, 4 and 6 as splints, in 150 μl of 50 mM Tris.Cl, pH 7.5, 10 mM $MgCl_2$. For the ligation, ATP (1 mM) and DTT (10mM) were added with 50 U T4 DNA ligase (Anglian Biotechnology Ltd.) and incubated for 30 minutes at room temperature. EDTA was added to 10 mM, the sample was extracted with phenol, precipitated from ethanol, dissolved in 20 μl water and boiled for 1 minute with an equal volume of formamide dyes. The sample was loaded onto and run on a 0.3 mm 8M-urea 10% polyacrylamide gel. A band of the expected size was detected by autoradiography and eluted by soaking.

Two full length single strands were assembled from blocks A to D and A' to D' using splint oligonucleotides. Thus blocks A to D were annealed and ligated in 30 μl as set out in the previous paragraph using 100 pmole of oligonucleotides 10a, 16 and 20 as splints. Blocks A' to D' were ligated using oligonucleotides 7, 13b and 17 as splints.

After phenol/ether extraction, block A-D was annealed with block A'-D', small amounts were cloned in the vector M13mp18 (Yanish-Perron, C., Vieira, J. and Messing, J., Gene, 33, 103-119, 1985) cut with PstI and Hind III, and the gene sequenced by the dideoxy technique (Sanger, F., Nicklen, S. and Coulson, A.R., PNAS USA, 74, 5463-5467, 1979).

The MoV_{NP} gene was transferred as a Hind III - BamHI fragment from the vector $pSV-V_{NP}$ (Neuberger et al., loc. cit.) to the vector M13mp8 (Messing, J. and Vieira, J., Gene, 19, 269-276, 1982). To facilitate the replacement of MoV_{NP} coding sequences by the synthetic HuV_{NP} fragment, three Hind II sites were removed from the 5' non-coding sequence by site directed mutagenesis, and a new Hind II site was subsequently introduced near the end of the fourth framework region (FR4 in Figure 2). By cutting the vector with PstI and Hind II, most of the V_{NP} fragment can be inserted as a PstI-Hind II fragment. The sequence at the Hind II site was corrected to NEWM FR4 by site directed mutagenesis.

The Hind III - Bam HI fragment, now carrying the HuV_{NP} gene, was excised from M13 and cloned back into $pSV-V_{NP}$ to replace the MoV_{NP} gene and produce a vector $pSV-HuV_{NP}$. Finally, the genes for the heavy chain constant domains of human Ig E (Flanagan, J.G. and Rabbitts, T.H., EMBO J., 1, 655-660, 1982) were introduced as a Bam HI fragment to give the vector $pSV-HuV_{NP}$. HE. This was transfected into the myeloma line J558 L by spheroplast fusion.

The sequence of the HuV_{NP} gene in $pSV-HuV_{NP}$. HE was checked by recloning the Hind III-Bam HI fragment back into M13mp8 (Messing et al., loc. cit.). J558L myeloma cells secrete lambda 1 light chains which have been shown to associate with heavy chains containing the MoV_{NP} variable domain to create a binding site for NP-cap or the related hapten NIP-Cap (3-iodo-4-hydroxy-5-nitrophenylacetyl-caproic acid) (Reth, M., Hammerling, G.J. and Rajewsky, K., Eur. J. Immunol., 8, 393-400, 1978).

As the plasmid pSV-HuV$_{NP}$.HE contains the gpt marker, stably transfected myeloma cells could be selected in a medium containing mycophenolic acid. Transfectants secreted an antibody (HuV$_{NP}$-IgE) with heavy chains comprising a HuV$_{NP}$ variable domain (i.e. a "humanised" mouse variable region) and human γ constant domains, and lambda 1 light chains from the J558L myeloma cells.

The culture supernatants of several gpt$^+$ clones were assayed by radioimmunoassay and found to contain NIP-cap binding antibody. The antibody secreted by one such clone was purified from culture supernatant by affinity chromatography on NIP-cap Sepharose (Sepharose is a registered trade mark). A polyacrylamide - SDS gel indicated that the protein was indistinguishable from the chimeric antibody MoV$_{NP}$-IgE (Neuberger et al., loc. cit.).

The HuV$_{NP}$-IgE antibody competes effectively with the MoV$_{NP}$-IgE for binding to both anti-human-IgE and to NIP-cap coupled to bovine serum albumin.

Various concentrations of HuV$_{NP}$-IgE and MoV$_{NP}$-IgE were used to compete the binding of radiolabelled MoV$_{NP}$-IgE to polyvinyl microtitre plates coated with (a) Sheep anti-human-IgE antiserum (Seward Laboratories); (b) NIP-cap-bovine serum albumin; (c) Ac38 anti-idiotypic antibody; (d) Ac 146 anti-idiotypic antibody; and (e) rabbit anti-MoV$_{NP}$

antiserum. Binding was also carried out in the presence of MoV_{NP}-IgM antibody (Neuberger, M.S., Williams, G.T. and Fox, R.O., Nature, 312, 604-608, 1984) or of JW5/1/2 which is an IgM antibody differing from the MoV_{NP}-IgM antibody at 13 residues mainly located in the V_H CDR2 region.

The results of the binding assays are shown in Figure 4, wherein black circles represent HuV_{NP}, white circles MoV_{NP}, black squares MoV_{NP}-IgM and white squares JW5/1/2. Binding is given relative to the binding in the absence of the inhibitor.

The affinities of HuV_{NP}-IgE for NP-cap and NIP-cap were then measured directly using the fluorescence quench technique and compared to those for MoVNP-IgE, using excitation at 295 nm and observing emission at 340 nm (Eisen, H.N., Methods Med. Res., 10, 115-121,1964).

Antibody solutions were diluted to 100 nM in phosphate buffered saline, filtered (0.45 µm pore cellulose acetate) and titrated with NP-cap in the range 0.2 to 20 µM. As a control, mouse DI-3 antibody (Mariuzza, R.A., Jankovic, D.L., Bulot, G., Amit, A.G., Saludjian, P., Le Guern, A., Mazie, J.C. and Poljak, R.J., J. Mol. Biol., 170, 1055-1058, 1983), which does not bind hapten, was titrated in parallel.

Decrease in the ratio of the fluorescence of HuV_{NP}-IgE or HuV_{NP}-IgE to the fluorescence of the D1-3 antibody was taken to be proportional to NP-cap occupancy of the antigen binding sites. The maximum quench was about 40% for both antibodies, and hapten dissociation constants were determined from least-squares fits of triplicate data sets to a hyperbola.

For NIP-cap, hapten concentration varied from 10 to 300 nM, and about 50% quenching of fluorescence was observed at saturation. Since the antibody concentrations were comparable to the value of the dissociation constants, data were fitted by least squares to an equation describing tight binding inhibition (Segal, I.H., in "Enzyme Kinetics", 73-74, Wiley, New York, 1975).

The binding constants obtained from these data for these antibodies are shown in Table 1 below.

Table 1

	$K_{NP\text{-}cap}$	$K_{NIP\text{-}cap}$
MoV_{NP}-IgE	1.2 µM	0.02 µM
HuV_{NP}-IgE	1.9 µM	0.07 µM

These results show that the affinities of these antibodies are similar and that the change in affinity is less than would be expected for the loss of a hydrogen bond or a van der Waals contact point at the active site of an enzyme.

Thus, it has been shown that it is possible to produce an antibody specific for an artificial small hapten, comprising a variable domain having human framework regions and mouse CDRs, without any significant loss of antigen binding capacity.

As shown in Figure 4(d), the HuV_{NP}-IgE antibody has lost the MoV_{NP} idiotypic determinant recognised by the antibody Ac146. Furthermore, HuV_{NP}-IgE also binds the Ac38 antibody less well (Figure 4(c)), and it is therefore not surprising that HuV_{NP}-IgE has lost many of the determinants recognised by the polyclonal rabbit anti-idiotypic antiserum (Figure 4(e)).

It can thus be seen that, although the HuV_{NP}-IgE antibody has acquired substantially all the antigen binding capacity of the mouse CDRs, it has not acquired any substantial proportion of the mouse antibody's antigenicity.

The results of Figures 4(d) and 4(e) carry a further practical implication. The mouse (or human) CDRs could be transferred from one set of human frameworks (antibody 1) to another (antibody 2). In therapy, anti-idiotypic antibodies generated in response to antibody 1 might well bind poorly to antibody 2. Thus, as the anti-idiotypic response starts to neutralise antibody 1 treatment could be continued with antibody 2, and the CDRs of a desired specificity used more than once.

For instance, the oligonucleotides encoding the CDRs may be used again, but with a set of oligonucleotides encoding a different set of framework regions.

The above work has shown that antigen binding characteristics can be transferred from one framework to another without loss of activity, so long as the original antibody is specific for a small hapten.

It is known that small haptens generally fit into an antigen binding cleft. However, this may not be true for natural antigens, for instance antigens comprising an epitopic site on a protein or polysaccharide. For such antigens, the antibody may lack a cleft (it may only have a shallow concavity), and surface amino acid residues may play a significant role in antigen binding. It is therefore not readily apparent that the work on artificial antigens shows conclusively that CDR replacement could be used to transfer natural antigen binding properties.

Therefore work was carried out to see if CDR replacement could be used for this purpose. This work also involved using primer-directed, oligonucleotide site-directed mutagenesis using three synthetic oligonucleotides coding for each of the mouse CDRs and the flanking parts of framework regions to produce a variable domain gene similar to the HuV_{NP} gene.

EXAMPLE 2

The three dimensional structure of a complex of lysozyme and the antilysozyme antibody D1.3 (Amit et al., loc. cit.) was solved by X-ray crystallography. There is a large surface of interaction between the antibody and antigen. The antibody has two heavy chains of the mouse IgG1 class (H) and two Kappa light chains (K), and is denoted below as H_2K_2.

The DNA sequence of the heavy chain variable region was determined by making cDNA from the mRNA of the D1.3 hybridoma cells, and cloning into plasmid and M13 vectors. The sequence is shown in Figure 7, in which the boxed residues comprise the three CDRs and the asterisks mark residues which contact lysozyme.

Three synthetic oligonucleotides were then designed to introduce the D1.3 V_HCDRs in place of the V_HCDRs of the HuV_{NP} gene. The Hu_{NP} gene has been cloned into M13mp8 as a BamHI-Hind III fragment, as described above. Each oligonucleotide has 12 nucleotides at the 5' end and 12 nucleotides at the 3' end which are complementary to the appropriate HuV_{NP} framework regions. The central portion of each oligonucleotide encodes either CDR1, CDR2, or CDR3 of the D1.3 antibody, as shown in Figure 5, to which reference is now made. It can be seen from this Figure that these oligonucleotides are 39, 72 and 48 nucleotides long respectively.

10 pmole of D1.3 CDR1 primer was phosphorylated at the 5' end and annealed to 1µg of the M13-HuV_{NP} template and extended with the Klenow fragment of DNA polymerase in the presence of T4 DNA ligase. After an oligonucleotide extension at 15°C, the sample was used to transfect E. Coli strain BHM71/18 mutL and plaques gridded and grown up as infected colonies.

After transfer to nitrocellulose filters, the colonies were probed at room temperature with 10 pmole of D1.3 CDR1 primer labelled at the 5' end with 30 μCi^{32}-p-ATP. After a 3" wash at 60°C, autoradiography revealed about 20% of the colonies had hybridised well to the probe. All these techniques are fully described in "Oligonucleotide site-directed mutagenesis in M13" an experimental manual by P. Carter, H. Bedouelle, M.M.Y. Waye and G. Winter 1985 and published by Anglian Biotechnology Limited, Hawkins Road, Colchester, Essex CO2 8JX. Several clones were sequenced, and the replacement of HuV_{NP} CDR1 by D.13 CDR1 was confirmed. This M13 template was used in a second round of mutagenesis with D1.3 CDR2 primer; finally template with both CDRs 1&2 replaced was used in a third round of mutagenesis with D.13 CDR3 primer. In this case, three rounds of mutagenesis were used.

The variable domain containing the D1.3 CDRs was then attached to sequences encoding the heavy chain constant regions of human IgG2 so as to produce a vector encoding a heavy chain Hu*. The vector was transfected into J558L cells as above. The antibody $Hu^*{}_2L_2$ is secreted.

For comparative purposes, the variable region gene for the D1.3 antibody was inserted into a suitable vector and attached to a gene encoding the constant regions of mouse IgG1 to produce a gene encoding a heavy chain H* with the same sequence as H. The protocol for achieving this is shown in Figure 8.

As shown in Figure 8, the gene encoding the D1.3 heavy chain V and C_H1 domains and part of the hinge region are cloned into the M13mp9 vector.

The vector (vector A) is then cut with NcoI, blunted with Klenow polymerase and cut with PstI. The PStI-NcoI fragment is purified and cloned into PstI-HindII cut MV_{NP} vector to replace most of the MV_{NP} coding sequences. The MV_{NP} vector comprises the mouse variable domain gene with its promoter, 5' leader, and 5' and 3' introns cloned into M13mp9. This product is shown as vector B in Figure 8.

Using site directed mutagenesis on the single stranded template of vector B with two primers, the sequence encoding the N-terminal portion of the C_H1 domain and the PstI site near the N-terminus of the V domain are removed. Thus the V domain of D1.3 now replaces that of V_{NP} to produce vector C of Figure 8.

Vector C is then cut with HindIII and BamHI and the fragment formed thereby is inserted into HindIII/BamHI cut M13mp9. The product is cut with Hind III and SacI and the fragment is inserted into PSV-V_{NP} cut with Hind III/SacI so as to replace the V_{NP} variable domain with the D1.3 variable domain. Mouse IgG1 constant domains are cloned into the vector as a SacI fragment to produce vector D of Figure 8.

Vector D of Figure 8 is transfected into J558L cells and the heavy chain H* is secreted in association with the lambda light chain L as an antibody $H^*{}_2L_2$.

Separated K or L light chains can be produced by treating an appropriate antibody (for instance D1.3 antibody to produce K light chains) with 2-mercaptoethanol in guanidine hydrochloride, blocking the free interchain sulphydryls with iodoacetamide and separating the dissociated heavy and light chains by HPLC in guanidine hydrochloride.

Different heavy and light chains can be reassociated to produce functional antibodies by mixing the separated heavy and light chains, and dialysing into a non-denaturing buffer to promote re-association and refolding. Properly reassociated and folded antibody molecules can be purified on protein A-sepharose columns. Using appropriate combinations of the above procedures, the following antibodies were prepared.

H_2K_2	(D1 3 antibody)
$H^*{}_2L_2$	(D1.3 heavy chain, lambda light chain)
$H^*{}_2K_2$	(recombinant equivalent of D1.3)
$Hu^*{}_2L_2$	("humanised" D1.3 heavy chain, lambda light chain)
$Hu^*{}_2K_2$	("humanised" D1.3)

The antibodies containing the lambda light chains were not tested for antigen binding capacity. The other antibodies were, and the results are shown in Table 2.

Table 2

Antibody	Dissociation constant for lysozyme (nM)
D1.3 (H_2K_2)	14.4
D1.3 (H_2K_2) (reassociated)	15.9, 11.4

recombinant D1.3 ($H^{*}{}_{2}K_{2}$) (reassociated)	9.2
"humanised" D1.3 ($Hu_{2}K_{2}$) (reassociated)	3.5, 3.7

The affinity of the antibodies for lysozyme was determined by fluorescent quenching, with excitation at 290nm and emission observed at 340nm Antibody solutions were diluted to 15-30µg/mg in phosphate buffered saline, filtered (0.45 um-cellulose acetate) and titrated with hen eggwhite lysozyme. There is a quenching of fluorescence on adding the lysozyme to the antibody (>100% quench) and data were fitted by least squares to an equation describing tight binding inhibition (I.H. Segal in Enzyme Kinetics, p73-74, Wiley, New York 1975). Although at first sight the data suggest that the binding of the "humanised" antibody to lysozyme is tighter than in the original D1.3 antibody, this remains to be confirmed. It is clear however that the humanised antibody binds lysozyme with a comparable affinity to D1.3

Further work (with another antibody-CAMPATH1) has shown that CDRs 1,2 and 3 can be exchanged simultaneously, by priming as above with all three primers. 10% hybridisation positives were detected by screening with the CDR1 primer; 30% of these comprised the triple mutant in which all the CDRs were replaced.

It has therefore been shown that CDR replacement can be used not only for artificial antigens (haptens) but also for natural antigens, thereby showing that the present invention will be of therapeutic use.

It will of course be understood that the present invention has been described above purely by way of example, and modifications of detail can be made within the scope of the invention as defined in the appended claims.

CLAIMS

1. An altered antibody in which at least parts of the complementarity determining regions (CDRs) in the light or heavy chain variable domains have been replaced by analogous parts of CDRs from an antibody of different specificity.

2. The altered antibody of claim 1, in which the entire CDRs have been replaced.

3. The altered antibody of claim 1 or claim 2, in which the variable domains in both the heavy and light chains have been altered by CDR replacement.

4. The altered antibody of any one of claims 1 to 3 in which the CDRs from a mouse antibody are grafted onto the framework regions of a human antibody.

5. The altered antibody of any one of claims 1 to 4, which has the structure of a natural antibody or a fragment thereof.

6. A method for producing an altered antibody comprising:

a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least a variable domain of an Ig heavy or light chain, the variable domain comprising framework regions from a first antibody and CDRs comprising at least parts of the CDRs from a second antibody of different specificity;

b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary Ig light or heavy chain respectively;

c) transforming a cell line with the first or both prepared vectors; and

d) culturing said transformed cell line to produce said altered antibody.

7. The method of claim 6, in which the cell line which is transformed to produce the altered antibody is an immortalised mammalian cell line.

8. The method of claim 7, in which the immortalised cell line is a myeloma cell line or a derivative thereof.

9. The method of any one of claims 6 to 8, in which the DNA sequence encoding the altered variable domain is prepared by oligonucleotide synthesis.

10. The method of any one of claims 6 to 8, in which the DNA sequence encoding the altered variable domain is prepared by primer directed oligonucleotide site-directed mutagenesis using a long oligonucleotide.

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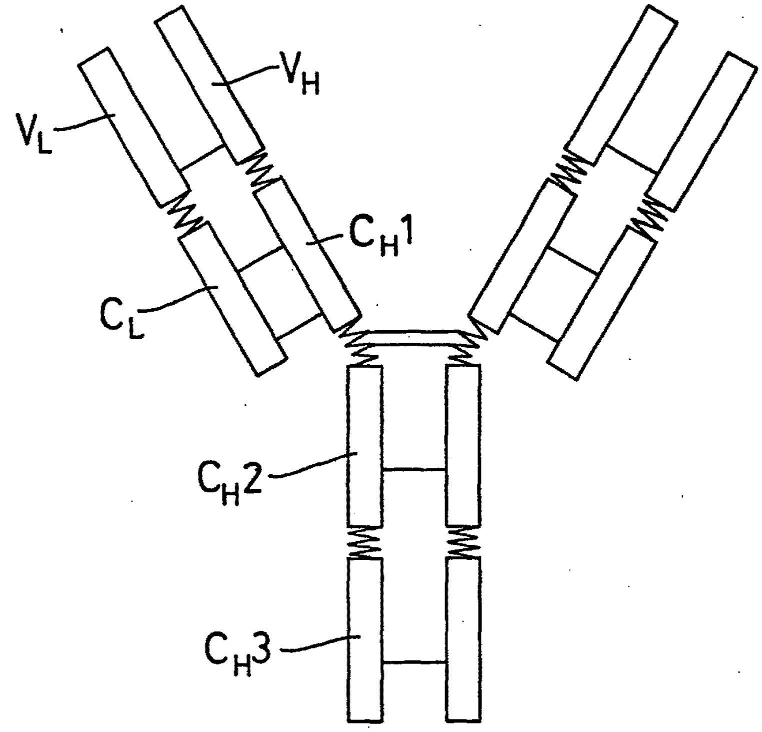
Fig. 1



▭ = domains
⋀⋀⋀ = inter-domain sections
—— = disulphide bonds
V = variable
C = constant
L = light chain
H = heavy chain

	1 FR1 30	31 CDR1 35
NEWM	XUQLQESGPGLURPSQTLSLTCTUSGSTFS	NDYYT
B1-8	QUQLQQPGAELUKPGASUKLSCKASGYTFT	SYWMH

	36 FR2 49	50 CDR2 65
NEWM	WURQPPGRGLEWIG	YUFYHGTSDDTTPLRS
B1-8	WUKQRPGRGLEWIG	RIDPNSGGTKYNEKFKS

	66 FR3 94	95 CDR3 102
NEWM	RUTMLUDTSKNQFSLRLSSUTAADTAUYYCAR	NLIAGCIDU
B1-8	KATLTUDKPSSTAYMQLSSLTSEDSAUYYCAR	YDYYGSSYFDY

	103 FR4 113
NEWM	WGQGSLUTUSS
B1-8	WGQGTTLTUSS

Fig. 2

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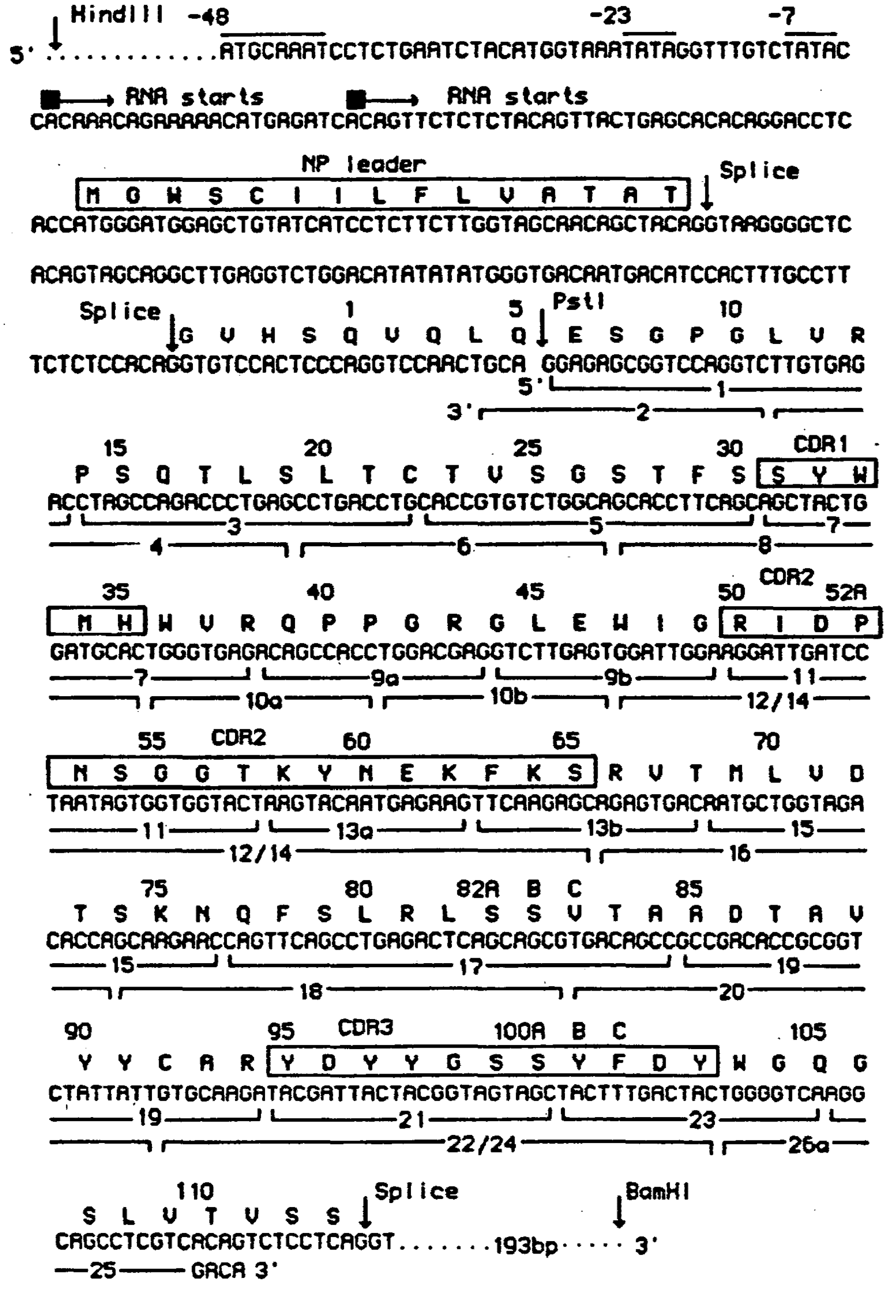
HindIII
-48
-23
-7
5' ............ATGCAAATCCTCTGAATCTACATGGTAAATATAGGTTTGTCTATAC
RNA starts
RNA starts
CACAAACAGAAAAACATGAGATCACAGTTCTCTCTACAGTTACTGAGCACACAGGACCTC
NP leader
M G W S C I I L F L V A T A T
Splice
ACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTAAGGGGCTC
ACAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTT
Splice
1
5
PstI
10
G V H S Q V Q L Q E S G P G L V R
TCTCTCCACAGGTGTCCACTCCCAGGTCCAACTGCA GGAGAGCGGTCCAGGTCTTGTGAG
5'
1
3'
2
15
20
25
30
CDR1
P S Q T L S L T C T V S G S T F S S Y W
ACCTAGCCAGACCCTGAGCCTGACCTGCACCGTGTCTGGCAGCACCTTCAGCAGCTACTG
3
5
7
4
6
8
35
40
45
50
CDR2
52A
M H W V R Q P P G R G L E W I G R I D P
GATGCACTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGAAGGATTGATCC
7
9a
9b
11
10a
10b
12/14
55
CDR2
60
65
70
N S G G T K Y N E K F K S R V T M L V D
TAATAGTGGTGGTACTAAGTACAATGAGAAGTTCAAGAGCAGAGTGACAATGCTGGTAGA
11
13a
13b
15
12/14
16
75
80
82A
B
C
85
T S K N Q F S L R L S S V T A A D T A V
CACCAGCAAGAACCAGTTCAGCCTGAGACTCAGCAGCGTGACAGCCGCCGACACCGCGGT
15
17
19
18
20
90
95
CDR3
100A
B
C
105
Y Y C A R Y D Y Y G S S Y F D Y W G Q G
CTATTATTGTGCAAGATACGATTACTACGGTAGTAGCTACTTTGACTACTGGGGTCAAGG
19
21
23
22/24
26a
110
Splice
BamHI
S L V T V S S
CAGCCTCGTCACAGTCTCCTCAGGT.......193bp.....3'
25
GACA 3'
26b
CTGTTCGA 5'

Fig. 3

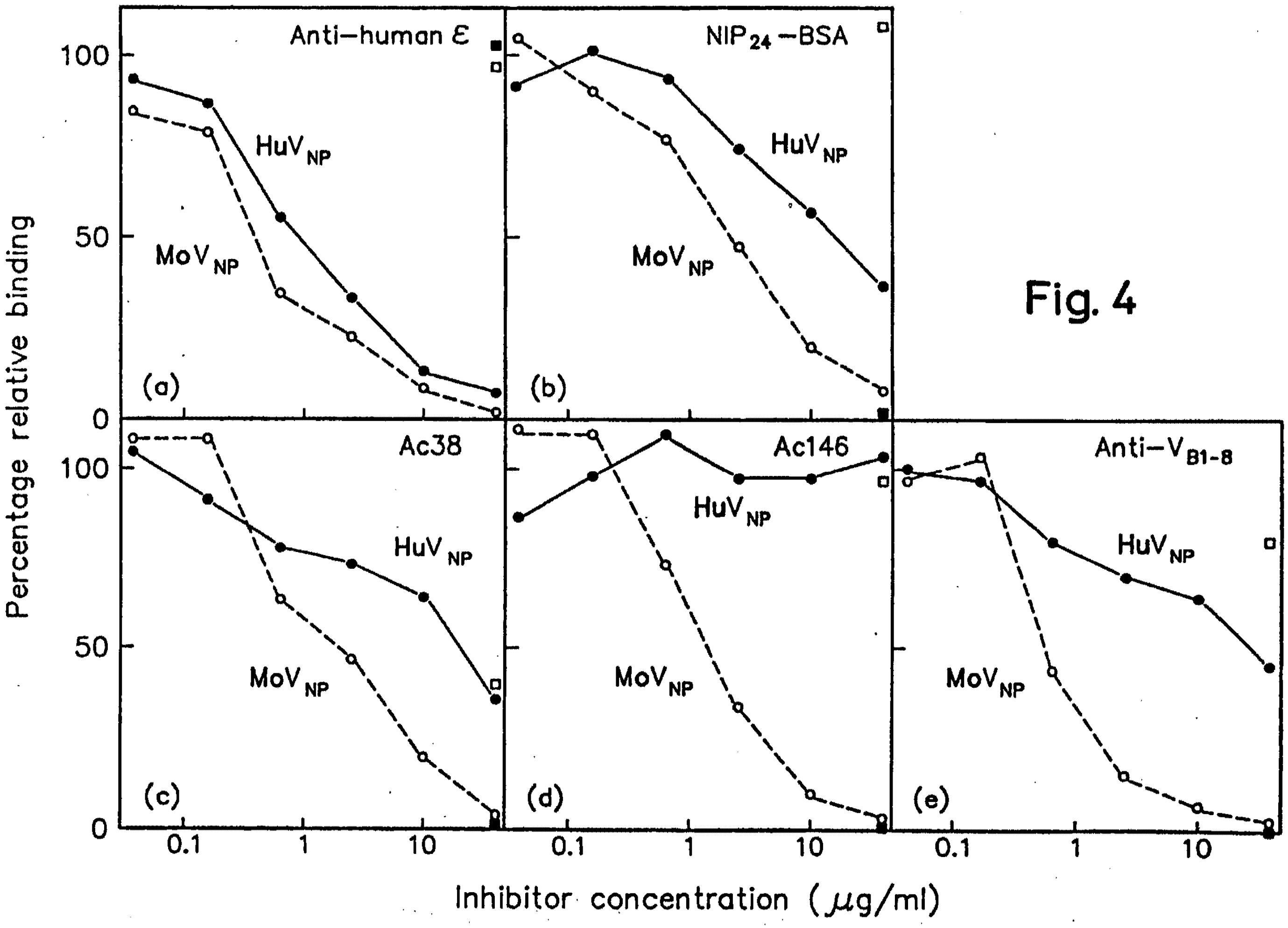
Anti–human ε
NIP$_{24}$–BSA
Ac38
Ac146
Anti–V$_{B1-8}$
HuV$_{NP}$
MoV$_{NP}$
(a)
(b)
(c)
(d)
(e)
Percentage relative binding
100
50
0
0.1
1
10
Inhibitor concentration (μg/ml)

Fig. 4

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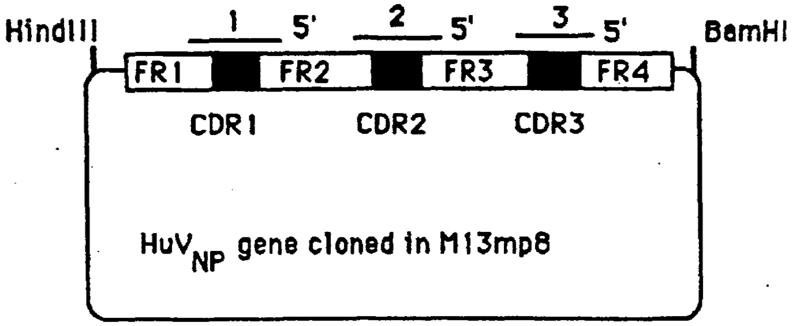
Fig. 5



D1.3 CDR1 oligonucleotide

5' CTG,TCT,CAC,CCA,GTT,TAC,ACC,ATA,GCC,GCT,GAA,GGT,GCT

FR2 D1.3 CDR1 FR1

D1.3 CDR2 oligonucleotide

5' CAT,TGT,CAC,TCT,GGA,TTT,GAG,AGC,TGA,ATT,ATA,GTC,TGT,

FR3 D1.3 CDR2

GTT,TCC,ATC,ACC,CCA,AAT,CAT,TCC,AAT,CCA,CTC

D1.3 CDR2 FR2

D1.3 CDR3 oligonucleotide

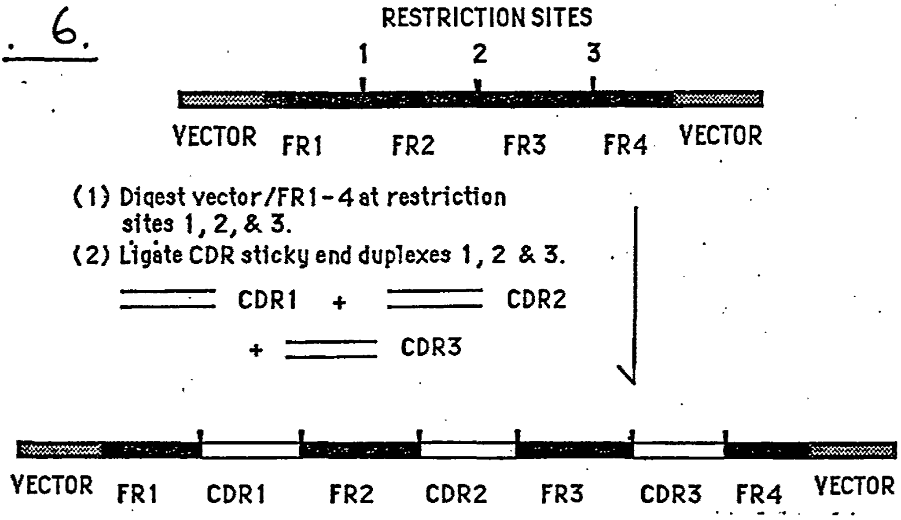
5' GCC,TTG,ACC,CCA,GTA,GTC,AAG,CCT,ATA,ATC,TCT,CTC,TCT,

FR4 D1.3 CDR3

TGC,ACA,ATA

FR3

Fig. 6.



(1) Digest vector/FR1-4 at restriction sites 1, 2, & 3.
(2) Ligate CDR sticky end duplexes 1, 2 & 3.

Fig. 7.

```
                -15 SIGNAL     -10            -5
        |M  A  V  L  A  L  L  F  C  L  V  T  F  P  S  C  I  L|
TCAGAGCATGGCTGTCCTGGCATTACTCTTCTGCCTGGTAACATTCCCAAGCTGTATCCT
 -1 +1          5              10             15
| S| Q  V  Q  L  K  E  S  G  P  G  L  V  A  P  S  Q  S  L  S
TTCCCAGGTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGCCTGTC
      [A]

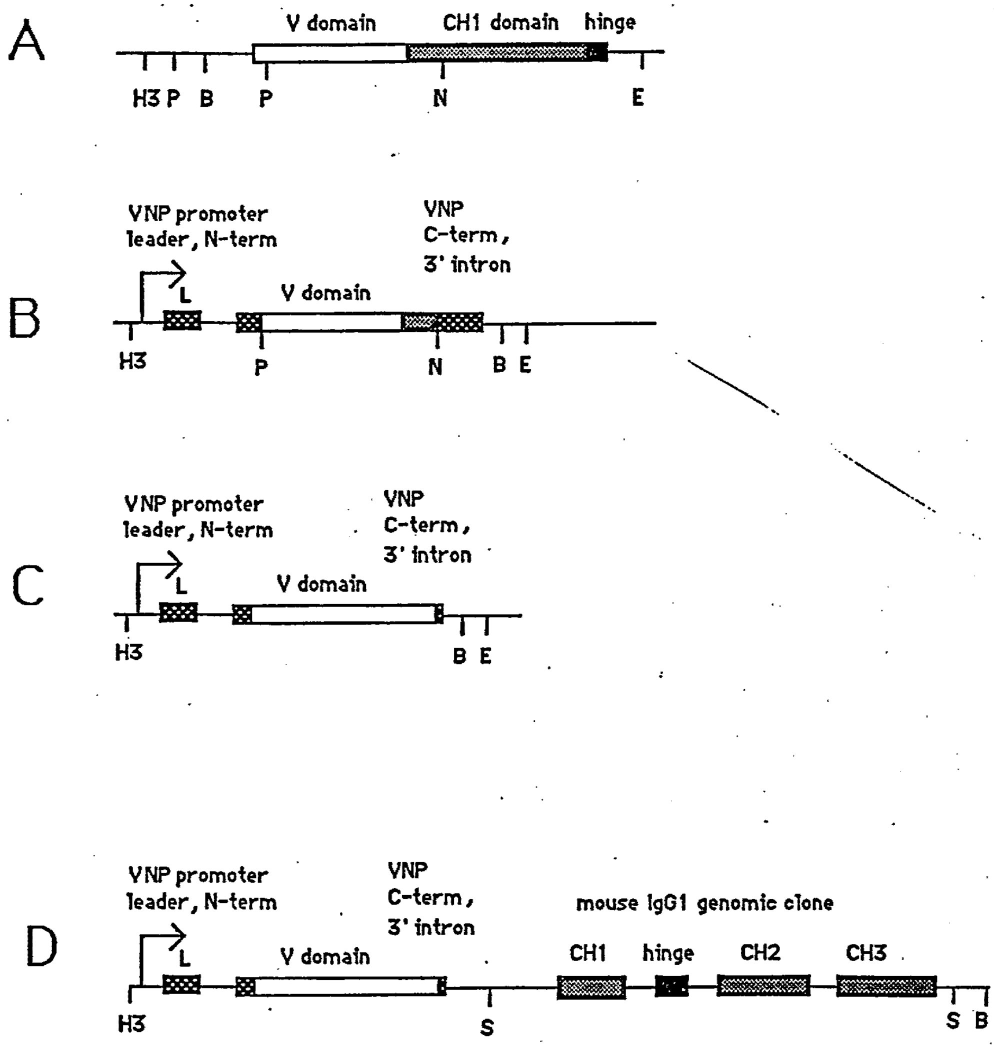
 20             25                 *  *  * CDR1 35
 I  T  C  T  V  S  G  F  S  L  T |G  Y  G  V  N| W  V  R  Q
CATCACATGCACCGTCTCAGGGTTCTCATTAACCGGCTATGGTGTAAACTGGGTTCGCCA

 40             45             50       *  *  * 55      CDR2
 P  P  G  K  G  L  E  W  L  G |M  I  W  G  D  G  N  T  D  Y|
GCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAATGATTTGGGGTGATGGAAACACAGACTA

 60    CDR2    65              70             75
|N  S  A  L  K  S| R  L  S  I  S  K  D  N  S  K  S  Q  V  F
TAATTCAGCTCTCAAATCCAGACTGAGCATCAGCAAGGACAACTCCAAGAGCCAAGTTTT
 80  .   82A  B  C  I     85             90             95  *
 L  K  M  N  S  L  H  T  D  D  T  A  R  Y  Y  C  A  R |E  R|
CTTAAAAATGAACAGTCTGCACACTGATGACACAGCCAGGTACTACTGTGCCAGAGAGAG
                                                      <-----

 *  *  *    CDR3         105            110
|D  Y  R  L  D  Y| W  G  Q  G  T  T  L  T  V  S  S
AGATTATAGGCTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
---- D ---><--------------- J_H2 --------------->
```

Fig. 8.



H3 = HindIII, P = PstI, B = BamHI, N = NcoI, E = EcoRI, H2 = HindII

⑲ Europäisches Patentamt
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⑪ Publication number: **0 239 400**
A3

⑫ EUROPEAN PATENT APPLICATION

㉑ Application number: **87302620.7**

㉒ Date of filing: **26.03.87**

(51) Int. Cl.⁴: **C 12 N 15/00**, C 07 K 15/06, C 12 P 21/02

㉚ Priority: **27.03.86 GB 8607679**

㊸ Date of publication of application: **30.09.87**
Bulletin 87/40

(84) Designated Contracting States: **AT BE CH DE ES FR GB GR IT LI LU NL SE**

(88) Date of deferred publication of search report: **05.04.89 Bulletin 89/14**

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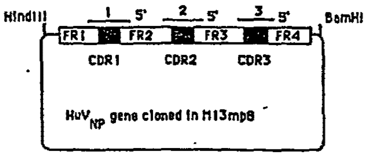
(54) Recombinant antibodies and methods for their production.

(57) An altered antibody is produced by replacing the complementarity determining regions (CDRs) of a variable region of an immunoglobulin (Ig) with the CDRs from an Ig of different specificity, using recombinant DNA techniques. The gene coding sequences for producing the altered antibody may be produced by site-directed mutagenesis using long oligonucleotides.



D1.3 CDR1 oligonucleotide
5' CTG,TCT,CAC,CCA,GTT,TAC,ACC,ATA,GCC,GCT,GAA,GGT,GCT
FR2 D1.3 CDR1 FR1

D1.3 CDR2 oligonucleotide
5' CAT,TGT,CAC,TCT,GGA,TTT,GAG,AGC,TGA,ATT,ATA,GTC,TGT,
FR3 D1.3 CDR2
GTT,TCC,ATC,ACC,CCA,AAT,CAT,TCC,AAT,CCA,CTC
D1.3 CDR2 FR2

D1.3 CDR3 oligonucleotide
5' GCC,TTG,ACC,CCA,GTA,GTC,AAG,CCT,ATA,ATC,TCT,CTC,TCT,
FR4 D1.3 CDR3
TGC,ACA,ATA
FR3

EP 0 239 400 A3

ACTORUM AG

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Office

EUROPEAN SEARCH REPORT

0239400
Application Number

EP 87 30 2620

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 82, April 1985, pages 2125-2127; P.P. CHEN et al.: "Possible involvement of human D minigenes in the first complementarity-determining region of K light chains" * Whole article * ---	1-10	C 12 N 15/00 C 07 K 15/06 C 12 P 21/02
X,P	NATURE, vol. 321, 29th May 1986, pages 522-525; P.T. JONES et al.: "Replacing the complementarity-determining regions in a human antibody with those from a mouse" * Whole article * ---	1-10	
T	SCIENCE, vol. 239, 25th March 1988, pages 1534-1536; M. VERHOEYEN et al.: "Reshaping human antibodies: grafting an antilysozyme activity" * Whole article * -----	1-10	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			C 12 N C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	26-01-1989	CUPIDO M.

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EPO FORM 1503 03.82 (P0401)

WO 90/07861(2) ( )
(C07K15/00H24H)-C07K15/00H3
[107K201:00]-[M07K203:00]-
[M07K207:00]-

-2- * - C07K15/00H34

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NDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: **C12P 21/00, C12N 5/10, 7/01, 15/00 ***	**A1**	**(11) International Publication Number:** **WO 90/07861** **(43) International Publication Date:** 26 July 1990 (26.07.90)

(21) International Application Number: PCT/US89/05857

(22) International Filing Date: 28 December 1989 (28.12.89)

(30) Priority data:
290,975 28 December 1988 (28.12.88) US
310,252 13 February 1989 (13.02.89) US

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(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CHIMERIC IMMUNOGLOBULINS SPECIFIC FOR p55 TAC PROTEIN OF THE IL-2 RECEPTOR

(57) Abstract

Novel methods for designing humanized immunoglobulins having one or more complementary determining regions (CDR's) from a donor immunoglobulin and a framework region from a human immunoglobulin comprising first comparing the framework or variable region amino acid sequence of the donor immunoglobulin to corresponding sequences in a collection of human immunoglobulin chains, and selecting as the human immunoglobulin one of the more homologous sequences from the collection. Each humanized immunoglobulin chain may comprise about 3 or more amino acids from the donor immunoglobulin in addition to the CDR's, usually at least one of which is immediately adjacent to a CDR in the donor immunoglobulin. The heavy and light chains may each be designed by using any one or all three additional position criteria. When combined into an intact antibody, the humanized immunoglobulins of the present invention will be substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin to the antigen, such as a protein or other compound containing an epitope.

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CHIMERIC IMMUNOGLOBULINS SPECIFIC FOR p55 TAC PROTEIN OF THE IL-2 RECEPTOR

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 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, such as those 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.

The present invention also provides novel methods for designing human-like immunoglobulin chains having one or more complementarity determining regions (CDR's) from a donor immunoglobulin and a framework region from a human immunoglobulin, the preferred methods comprising first comparing the framework or variable region amino acid sequence of the donor immunoglobulin to corresponding sequences in a collection of human immunoglobulin chains, and selecting as the human immunoglobulin one of the more homologous sequences from the collection. The human immunoglobulin, or acceptor immunoglobulin, sequence is typically selected from a collection of at least 10 to 20 immunoglobulin chain sequences, and usually will have the highest homology to the donor immunoglobulin sequence of any sequence in the collection. The human immunoglobulin framework sequence will typically have about 65 to 70% homology or more to the donor immunoglobulin framework sequences. The donor immunoglobulin may be either a heavy chain or light chain (or both), and the human collection will contain the same kind of chain. A humanized light and heavy chain can be used to form a complete humanized immunoglobulin or antibody, having two light/heavy chain pairs, with or without partial or full-length human constant regions and other proteins.

In another embodiment of the present invention, either in conjunction with the above comparison step or separately, additional amino acids in an acceptor immunoglobulin chain may be replaced with amino acids form the CDR-donor immunoglobulin chain. More specifically, further optional substitutions of a human framework amino acid of the acceptor immunoglobulin with a corresponding amino acid from a donor immunoglobulin will be made at positions in the immunoglobulins where:

(a) the amino acid in the human framework region of an acceptor immunoglobulin is rare for that position and the corresponding amino acid in the donor immunoglobulin is common for that position in human immunoglobulin sequences; or

(b) the amino acid is immediately adjacent to one of the CDR's; or

(c) the amino acid is predicted to be within about 3Å of the CDR's in a three-dimensional immunoglobulin model and capable of interacting with the antigen or with the CDR's of the humanized immunoglobulin.

The humanized immunoglobulin chain will typically comprise at least about 3 amino acids from the donor immunoglobulin in addition to the CDR's, usually at least one of which is immediately adjacent to a CDR in the donor immunoglobulin. The heavy and light chains may each be designed by using any one or all three of the position criteria.

When combined into an intact antibody, the humanized light and heavy chains of the present invention will be substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin to the antigen (such as a protein or other compound containing an epitope). These affinity levels can vary from about 10^8 M^{-1} or higher, and may be within about 4 fold of the donor immunoglobulin's original affinity to the antigen.

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 synthesize 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 one embodiment of the present invention, human-like immunoglobulins specifically reactive with desired epitopes, such as those on 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 a human immunoglobulin.

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.

In accordance with another general aspect of the present invention, also included are criteria by which a limited number of amino acids in the framework of a human-like or humanized immunoglobulin chain are chosen to be the same as the amino acids at those positions in the donor Ig rather than in the acceptor Ig, in order to increase the affinity of an antibody comprising the humanized immunoglobulin chain.

This aspect of the present invention is based in part on the model that two contributing causes of the loss of affinity in prior means of producing humanized antibodies (using as examples mouse antibodies as the source of CDR's) are:

(1) When the mouse CDR's are combined with the human framework, the amino acids in the framework close to the CDR's become human instead of mouse. Without intending to be bound by theory, we believe that these changed amino acids may slightly distort the CDR's, because they create different electrostatic or hydrophobic forces than in the donor mouse antibody, and the distorted CDR's may not make as

effective contacts with the antigen as the CDR's did in the donor antibody;

(2) Also, amino acids in the original mouse antibody that are close to, but not part of, the CDR's (i.e., still part of the framework), may make contacts with the antigen that contribute to affinity. These amino acids are lost when the antibody is humanized, because all framework amino acids are made human.

To avoid these problems, and to produce humanized antibodies that have a very strong affinity for a desired antigen, the present invention uses the following four criteria for designing humanized immunoglobulins. These criteria may be used singly, or when necessary in combination, to achieve the desired affinity or other characteristics.

Criterion I: As acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. For example, comparison of the sequence of a mouse heavy (or light) chain variable region against human heavy (or light) variable regions in a data bank (for example, the National Biomedical Research Foundation Protein Identification Resource) shows that the extent of homology to different human regions varies greatly, typically from about 40% to about 60-70%. By choosing as the acceptor immunoglobulin one of the human heavy (respectively light) chain variable regions that is most homologous to the heavy (respectively light) chain variable region of the donor immunoglobulin, fewer amino acids will be changed in going from the donor immunoglobulin to the humanized immunoglobulin. Hence, and again without intending to be bound by theory, it is believed that there is a smaller chance of changing an amino acid near the CDR's that distorts their conformation. Moreover, the precise overall shape of a humanized antibody comprising the humanized immunoglobulin chain may more closely resemble the shape of the donor antibody, also reducing the chance of distorting the CDR's.

Typically, one of the 3-5 most homologous heavy chain variable region sequences in a representative collection of at least about 10 to 20 distinct human heavy chains will be chosen as acceptor to provide the heavy chain framework, and similarly for the light chain. Preferably, one of the 1-3 most homologous variable regions will be used. The selected acceptor immunoglobulin chain will most preferably have at least about 65% homology in the framework region to the donor immunoglobulin.

Regardless of how the acceptor immunoglobulin is chosen, higher affinity may be achieved by selecting a small number of amino acids in the framework of the humanized immunoglobulin chain to be the same as the amino acids at those positions in the donor rather than in the acceptor. The following criteria define what amino acids may be so selected. Preferably, at most or all amino acid positions satisfying one of these criteria, the donor amino acid will in fact be selected.

Criterion II: If an amino acid in the framework of the human acceptor immunoglobulin is unusual (i.e., "rare", which as used herein indicates an amino acid occurring at that position in no more than about 10% of human heavy (respectively light) chain V region sequences in a representative data bank), and if the donor amino acid at that position is typical for human sequences (i.e., "common", which as used herein indicates an amino acid occurring in at least about 25% of sequences in a representative data bank), then the donor amino acid rather than the acceptor may be selected. This criterion helps ensure that an atypical amino acid in the human framework does not disrupt the antibody structure. Moreover, by replacing an unusual amino acid with an amino acid from the donor antibody that happens to be typical for human antibodies, the humanized antibody may be made less immunogenic.

Criterion III: In the positions immediately adjacent to the 3 CDR's in the humanized immunoglobulin chain, the donor amino acid rather than acceptor amino acid may be selected. These amino acids are particularly likely to interact with the amino acids in the CDR's and, if chosen from the acceptor, distort the donor CDR's and reduce affinity. Moreover, the adjacent amino acids may interact directly with the antigen (Amit _et al._, _Science_, _233_, 747-753 (1986), which is incorporated herein by reference) and selecting these amino acids from the donor may be desirable to keep all the antigen contacts that provide affinity in the original antibody.

Criterion IV: A 3-dimensional model, typically of the original donor antibody, shows that certain amino acids outside of the CDR's are close to the CDR's and have a good probability of interacting with amino acids in the CDR's by hydrogen bonding, Van der Waals forces, hydrophobic interactions, etc. At those amino acid positions, the donor amino acid rather than the acceptor immunoglobulin amino acid may be selected. Amino acids according to this criterion will generally have a side chain atom within about 3 angstrom units of some site in the CDR's and must contain atoms that could interact with the CDR atoms according to established chemical forces, such as those listed above. Computer programs to create models of proteins such as antibodies are generally available and well known to those skilled in the art (_see_, Loew _et al._, _Int. J. Quant. Chem._, _Quant. Biol. Symp._, _15_:55-66 (1988); Bruccoleri _et al._, _Nature_, _335_, 564-568 (1988); Chothia _et al._, _Science_, _233_:755-758 (1986), all of which are incorporated herein by reference). These do not form part of the invention. Indeed, because all antibodies have similar structures, the known antibody structures, which are available from the Brookhaven Protein Data Bank, can be used if necessary as rough models of other antibodies. Commercially available computer programs can be used to display these models on a computer monitor, to calculate the distance between atoms, and to estimate the likelihood of different amino acids interacting (see, Ferrin et al., J. Mol. Graphics, 6:13-27 (1988)).

Humanized or human-like antibodies generally have at least three potential advantages over mouse or 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 constant region of the humanized 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 (D. Shaw et al., J. Immunol., 138:4534-4538 (1987)). Injected humanized antibodies will presumably have a half-life more similar to naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

The present invention is specifically directed to improved humanized immmunoglobulins (e.g., capable of binding the human IL-2 receptor) with respect to those described in EPA publication no. 0239400. That application, the disclosure of which is excluded from coverage herein, describes, for certain immunoglobulins, substituting CDR's regions in the light or heavy chain variable domains of an acceptor antibody with analogous parts of CDR's (typically solvent accessible) from an antibody of different specificity. Also, that application discusses, for certain immunoglobulins, the possibility of only transferring residues that are (solvent) accessible from the antigen binding site, which residues apparently may include certain framework regions (specifically, residues known to be involved in antigen binding as described in Amit et al., Science 233: 747-753 (1986) or perhaps residues essential for inter-chain interactions - but for the selection of which insufficient guidance is provided in that application). Thus, for example, a preferred embodiment of the present invention entails substituting entire CDR's and framework amino acids immediately adjacent one (or preferably each) of the CDR's. In general, any framework residue that also makes contact with the CDR's to, e.g., maintain their conformation (and usually their antigen binding specificity) are specifically included within preferred embodiments of the present invention as described in detail, supra.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDR's (typically with other amino acid residues as described above) from an immunoglobulin capable of binding to a desired epitope, such as on the human IL-2 receptor (e.g., the anti-Tac monoclonal antibody). The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate human-like framework regions. For example, 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 3 and 4, 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). For example, the human kappa immunoglobulin constant and J region genes and sequences are described in Heiter et al., Cell 22:197-207 (1980) and the nucleotide sequence of a human immunoglobulin $C_{\gamma 1}$ gene is described in Ellison et al., Nucl. Acid. Res. 10:4071 (1982), both of which are incorporated herein by reference. The CDR's for producing the immunoglobulins of the present invention will be similarly derived from monoclonal antibodies capable of binding to the desired antigen (e.g., 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, for the IL-2 receptor immunoglobulins 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 SV40 with enhancer (see, Mulligan and Berg, Science 209:1422-1427 (1980), an immunglobulin gene, 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 IL-2 receptor specific antibodies exemplified in 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. 07/290,968 filed December 28, 1988), "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 in vitro. By way of example, the exemplary 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 co-factors, 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 x 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 (within 3 to 4 fold), because if one had much greater affinity, it would have more effectively competed with the biotinylated anti-Tac, thus decreasing fluorescence more.

Biological properties of the humanized antibody

For optimal use in treatment of human disease, the humanized antibody should be able to destroy T-cells in the body that express the IL-2 receptor. One mechanism by which antibodies may destroy target cells is antibody-dependent cell-mediated cytotoxicity, abbreviated ADCC (Fundamental Immunology, Paul, W., Ed., Raven Press, New York (1984), at pg. 681), in which the antibody forms a bridge between the target cell and an effector cell such as a macrophage that can lyse the target. To determine whether the humanized antibody and the original mouse anti-Tac antibody can mediate ADCC, a chromium release assay was performed by standard methods. Specifically, human leukemia HUT-102 cells, which express the IL-2 receptor, were incubated with ^{51}Cr to allow them to absorb this radionuclide. The HUT-102 cells were then incubated with an excess of either anti-Tac or humanized anti-Tac antibody. The HUT-102 cells were next incubated for 4 hrs with either a 30:1 or 100:1 ratio of effector cells, which were normal purified human peripheral blood mononuclear cells that had been activated by incubation for about 20 hrs with human recombinant IL-2. Release of ^{51}Cr, which indicated lysis of the target HUT-102 cells, was measured and the background subtracted (Table 1). The results show that at either ratio of effector cells, anti-Tac did not lyse a significant number of the target cells (less than 5%), while the humanized antibody did (more than 20%). Hence, the humanized antibody is likely to be more efficacious than the original mouse antibody in treating T-cell leukemia or other T-cell mediated diseases.

TABLE 1

Percent ^{51}Cr release after ADCC

	Effector: Target ratio	
	30:1	100:1
Antibody		
Anti-Tac	4%	< 1%
Humanized anti-Tac	24%	23%

From the foregoing, it will be appreciated that the human-like immunoglobulins of the present invention offer numerous advantages of other antibodies. For example, in comparison to anti-Tac mouse monoclonal antibodies, the present human-like IL-2 receptor immunoglobulins can be more economically produced and contain substantially less foreign amino acid sequences. This reduced likelihood of antigenicity after injection into a human patient represents a significant therapeutic improvement for immunoglobulins designed in accordance with the above criteria.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WE CLAIM:

1. A composition comprising a substantially pure human-like immunoglobulin specifically reactive with p55 Tac protein.

2. A composition according to Claim 1, wherein the immunoglobulin comprises two pairs of light/heavy chain dimers, wherein each chain comprises a variable region and a constant region.

3. A composition comprising a substantially pure human-like immunoglobulin capable of inhibiting binding of human interleukin-2 (IL-2) to a human IL-2 receptor.

4. A composition according to Claim 1, wherein the immunoglobulin exhibits a binding affinity to a human IL-2 receptor of about 10^8 M^{-1} or stronger.

5. A composition according to Claim 1, wherein the immunoglobulin comprises complementarity determining regions from one immunoglobulin and framework regions from at least one different immunoglobulin.

6. A recombinant immunoglobulin composition comprising a human-like framework and one or more foreign complementarity determining regions not naturally associated with the framework, wherein said immunoglobulin is capable of binding to a human interleukin-2 receptor.

7. A composition according to Claim 6, wherein the immunoglobulin is an IgG_1 immunoglobulin isotype.

8. A composition according to Claim 6, wherein the mature light and heavy variable region protein sequences are substantially homologous to the mature protein sequences in Figures 3 and 4.

9. A human-like immunoglobulin having two pairs of light chain/heavy chain dimers and capable of specifically reacting with an epitope on a human interleukin-2 receptor with an affinity of at least about 10^8 M^{-1}, said light and heavy chains comprising complementarity determining regions (CDR's) and human-like framework regions, wherein the CDR's are from different immunoglobulin molecules than the framework regions.

10. An immunoglobulin according to Claim 9, which is capable of blocking the binding of interleukin-2 (IL-2) to human IL-2 receptors.

11. A humanized immunoglobulin capable of binding to human interleukin-2 receptors, said immunoglobulin comprising one or more complementarity determining regions (CDR's) from anti-Tac antibody in a human-like framework, wherein the human-like framework region comprises at least one amino acid chosen from the anti-Tac antibody.

12. A humanized immunoglobulin according to Claim 11, having a mature heavy chain variable sequence as shown in Figure 3, and a mature light chain sequence as shown in Figure 4.

13. A humanized immunoglobulin according to Claim 11, wherein an additional amino acid from the anti-Tac antibody is immediately adjacent a CDR.

14. A method of treating T-cell mediated disorders in a human patient, said method comprising administering to said patient a therapeutically effective dose of an immunoglobulin according to Claim 1.

15. An immunoglobulin according to Claim 1 which was produced in a myeloma or hybridoma cell.

16. A polynucleotide molecule comprising a first sequence coding for human-like immunoglobulin framework regions and a second sequence coding for one or more mouse immunoglobulin complementarity determining regions, wherein upon expression said polynucleotide encodes an immunoglobulin specifically reactive with p55 Tac protein and capable of blocking the binding of interleukin-2 (IL-2) to the IL-2 receptor on human T-cells.

17. A cell line transfected with a polynucleotide of Claim 16.

18. A method of designing a humanized immunoglobulin (Ig) chain having one or more complementarity determining regions (CDR's) from a donor Ig and a framework region from a human Ig, said method comprising: comparing the framework or variable region amino acid sequence of the donor Ig light or heavy chain with corresponding sequences in a collection of human Ig chains; and selecting to provide the human Ig light or heavy chain framework one of the about three most homologous sequences from the collection.

19. A method of designing a humanized immunoglobulin chain having a framework region from a human acceptor immunoglobulin and complementarity determining regions (CDR's) from a donor immunoglobulin capable of binding to an antigen, said method comprising the steps of substituting at least one human framework amino acid of the acceptor immunoglobulin with a corresponding amino acid from the donor immunoglobulin at a position in the immunoglobulins where:

(a) the amino acid in the human framework region of the acceptor immunoglobulin is rare for said position and the corresponding amino acid in the donor immunoglobulin is common for said position in human immunoglobulin sequences; or

(b) the amino acid is immediately adjacent to one of the CDR's; or

(c) the amino acid is predicted to have a side chain atom within about 3Å of the CDR's in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDR's of the humanized immunoglobulin.

20. A method according to Claim 19, wherein the humanized immunoglobulin chain comprises in addition to the CDR's at least three amino acids from the donor immunoglobulin chosen by criteria (a), (b) or (c).

21. A method according to Claim 20, wherein at least one of the amino acids substituted from the donor is immediately adjacent a CDR.

22. A humanized immunoglobulin designed according to Claims 18, 19, or 20.

```
1     Q  V  Q  L  Q  Q  S  G  A  E  L  A  K  P  G  A  S  V  K  M
      |  |  |  |     |  |  |  |  |        |  |  |     |  |  |
1     Q  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  S  S  V  K  V

21    S  C  K  A  S  G  Y  T  F  T  S  Y  R  M  H  W  V  K  Q  R
      |  |  |  |  |  |     |  |                    |  |     |
21    S  C  K  A  S  G  G  T  F  S  R  S  A  I  I  W  V  R  Q  A
                        *        *  -------------

41    P  G  Q  G  L  E  W  I  G  Y  I  N  P  S  T  G  Y  T  E  Y
      |  |  |  |  |  |  |     |     |     |        |           |
41    P  G  Q  G  L  E  W  M  G  G  I  V  P  M  F  G  P  P  N  Y
                           *     -------------------------------

61    N  Q  K  F  K  D  K  A  T  L  T  A  D  K  S  S  S  T  A  Y
         |  |  |              |     |  |  |     |        |  |  |
61    A  Q  K  F  Q  G  R  V  T  I  T  A  D  E  S  T  N  T  A  Y
      ----------------- *  *

81    M  Q  L  S  S  L  T  F  E  D  S  A  V  Y  Y  C  A  R  G
      |     |  |  |  |        |  |     |     |     |  |     |
81    M  E  L  S  S  L  R  S  E  D  T  A  F  Y  F  C  A  G  G  Y
                                          *     *        *  -----

100   G  G  V  F  D  Y  W  G  Q  G  T  T  L  T  V  S  S
      |                          |           |  |  |  |
101   G  I  Y  S  P  E  E  Y  N  G  G  L  V  T  V  S  S
      ----------------- *  *  *     *
```

FIG._1.

```
1     Q  I  V  L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K  V  T
         |        |  |  |  |           |  |  |     |        |  |
1     D  I  Q  M  T  Q  S  P  S  T  L  S  A  S  V  G  D  R  V  T

21    I  T  C  S  A  S  S  S  I     S  Y  M  H  W  F  Q  Q  K  P
      |  |  |     |  |     |  |                 |     |  |  |  |
21    I  T  C  R  A  S  Q  S  I  N  T  W  L  A  W  Y  Q  Q  K  P
               --------------------------------

40    G  T  S  P  K  L  W  I  Y  T  T  S  N  L  A  S  G  V  P  A
      |        |  |  |        |        |     |     |  |  |  |
41    G  K  A  P  K  L  L  M  Y  K  A  S  S  L  E  S  G  V  P  S
                           *     --------------------          *

60    R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I  S  R  M  E  A
      |  |     |  |  |  |  |  |           |  |  |  |
61    R  F  I  G  S  G  S  G  T  E  F  T  L  T  I  S  S  L  Q  P
            *

80    E  D  A  A  T  Y  Y  C  H  Q  R  S  T  Y  P  L  T  F  G  S
         |     |  |  |  |  |     |                       |  |
81    D  D  F  A  T  Y  Y  C  Q  Q  Y  N  S  D  S  K  M  F  G  Q
                              --------------------------

100   G  T  K  L  E  L  K
      |  |  |     |     |
101   G  T  K  V  E  V  K
```

FIG 2

```
        10        20        30        40        50        60
TCTAGATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGTACCGCGGGCGTGCACT
      M  G  W  S  W  I  F  L  F  L  L  S  G  T  A  G  V  H

        70        80        90       100       110       120
CTCAGGTCCAGCTTGTCCAGTCTGGGGCTGAAGTCAAGAAACCTGGCTCGAGCGTGAAGG
S  Q  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  S  S  V  K

       130       140       150       160       170       180
TCTCCTGCAAGGCTTCTGGCTACACCTTTACTAGCTACAGGATGCACTGGGTAAGGCAGG
V  S  C  K  A  S  G  Y  T  F  T  S  Y  R  M  H  W  V  R  Q

       190       200       210       220       230       240
CCCCTGGACAGGGTCTGGAATGGATTGGATATATTAATCCGTCGACTGGGTATACTGAAT
A  P  G  Q  G  L  E  W  I  G  Y  I  N  P  S  T  G  Y  T  E

       250       260       270       280       290       300
ACAATCAGAAGTTCAAGGACAAGGCAACAATTACTGCAGACGAATCCACCAATACAGCCT
Y  N  Q  K  F  K  D  K  A  T  I  T  A  D  E  S  T  N  T  A

       310       320       330       340       350       360
ACATGGAACTGAGCAGCCTGAGATCTGAGGACACCGCAGTCTATTACTGTGCAAGAGGGG
Y  M  E  L  S  S  L  R  S  E  D  T  A  V  Y  Y  C  A  R  G

       370       380       390       400       410       420
GGGGGGTCTTTGACTACTGGGGCCAAGGAACCCTGGTCACAGTCTCCTCAGGTGAGTCCT
G  G  V  F  D  Y  W  G  Q  G  T  L  V  T  V  S  S

       430
TAAAACCTCTAGA
```

FIG._3.

```
        10        20        30        40        50        60
TCTAGATGGAGACCGATACCCTCCTGCTATGGGTCCTCCTGCTATGGGTCCCAGGATCAA
      M  E  T  D  T  L  L  L  W  V  L  L  L  W  V  P  G  S

        70        80        90       100       110       120
CCGGAGATATTCAGATGACCCAGTCTCCATCTACCCTCTCTGCTAGCGTCGGGGATAGGG
T  G  D  I  Q  M  T  Q  S  P  S  T  L  S  A  S  V  G  D  R

       130       140       150       160       170       180
TCACCATAACCTGCTCTGCCAGCTCAAGTATAAGTTACATGCACTGGTACCAGCAGAAGC
V  T  I  T  C  S  A  S  S  S  I  S  Y  M  H  W  Y  Q  Q  K

       190       200       210       220       230       240
CAGGCAAAGCTCCCAAGCTTCTAATTTATACCACATCCAACCTGGCTTCTGGAGTCCCTG
P  G  K  A  P  K  L  L  I  Y  T  T  S  N  L  A  S  G  V  P

       250       260       270       280       290       300
CTCGCTTCAGTGGCAGTGGATCTGGGACCGAGTTCACCCTCACAATCAGCTCTCTGCAGC
A  R  F  S  G  S  G  S  G  T  E  F  T  L  T  I  S  S  L  Q

       310       320       330       340       350       360
CAGATGATTTCGCCACTTATTACTGCCATCAAAGGAGTACTTACCCACTCACGTTCGGTC
P  D  D  F  A  T  Y  Y  C  H  Q  R  S  T  Y  P  L  T  F  G

       370       380       390       400
AGGGGACCAAGGTGGAGGTCAAACGTAAGTACACTTTTCTAGA
Q  G  T  K  V  E  V  K
```

FIG._4.

A

HES12 AGCTTCTAGATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGTACCGCGGGCGTG
CACTCTCAGGTCCAGCTTGTCCAGTCTGGGGCTGAAGTCAAGAAACCTGGCTCGAGCGTG
AAGGTC

HES13 GCCAGTCGACGGATTAATATATCCAATCCATTCCAGACCCTGTCCAGGGGCCTGCCTTAC
CCAGTGCATCCTGTAGCTAGTAAAGGTGTAGCCAGAAGCCTTGCAGGAGACCTTCACGCT
CGAGCCAGG

HES14 TATATTAATCCGTCGACTGGGTATACTGAATACAATCAGAAGTTCAAGGACAAGGCAACA
ATTACTGCAGACGAATCCACCAATACAGCCTACATGGAACTGAGCAGCCTGAGATCTGAG
GACA

HES15 ATATCGTCTAGAGGTTTTAAGGACTCACCTGAGGAGACTGTGACCAGGGTTCCTTGGCCC
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AGGCTGCT

B

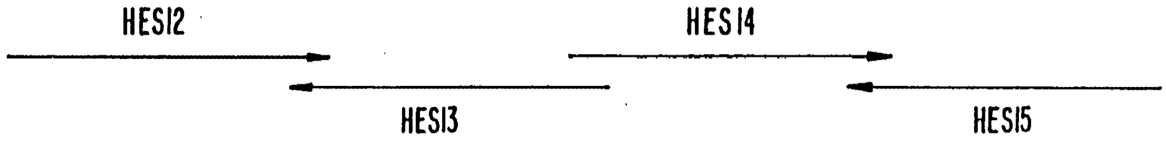

FIG._5.

A

JFD1 CAAATCTAGATGGAGACCGATACCCTCCTGCTATGGGTCCTCCTGCTATGGGTCCCAGGA
TCAACCGGAGATATTCAGATGACCCAGTCTCCATCTACCCTCTCTGCTAGCGTCGGGGAT

JFD2 ATAAATTAGAAGCTTGGGAGCTTTGCCTGGCTTCTGCTGGTACCAGTGCATGTAACTTAT
ACTTGAGCTGGCAGAGCAGGTTATGGTGACCCTATCCCCGACGCTAGCAGAGAG

JFD3 GCTCCCAAGCTTCTAATTTATACCACATCCAACCTGGCTTCTGGAGTCCCTGCTCGCTTC
AGTGGCAGTGGATCTGGGACCGAGTTCACCCTCACAATCAGCTCTCTGCAGCCAGATGAT
TTC

JFD4 TATATCTAGAAAAGTGTACTTACGTTTGACCTCCACCTTGGTCCCCTGACCGAACGTGAG
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GA

B

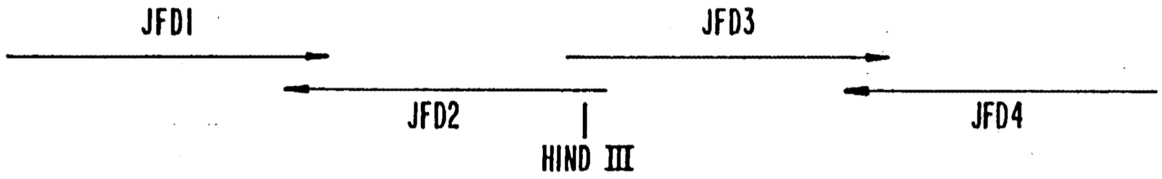
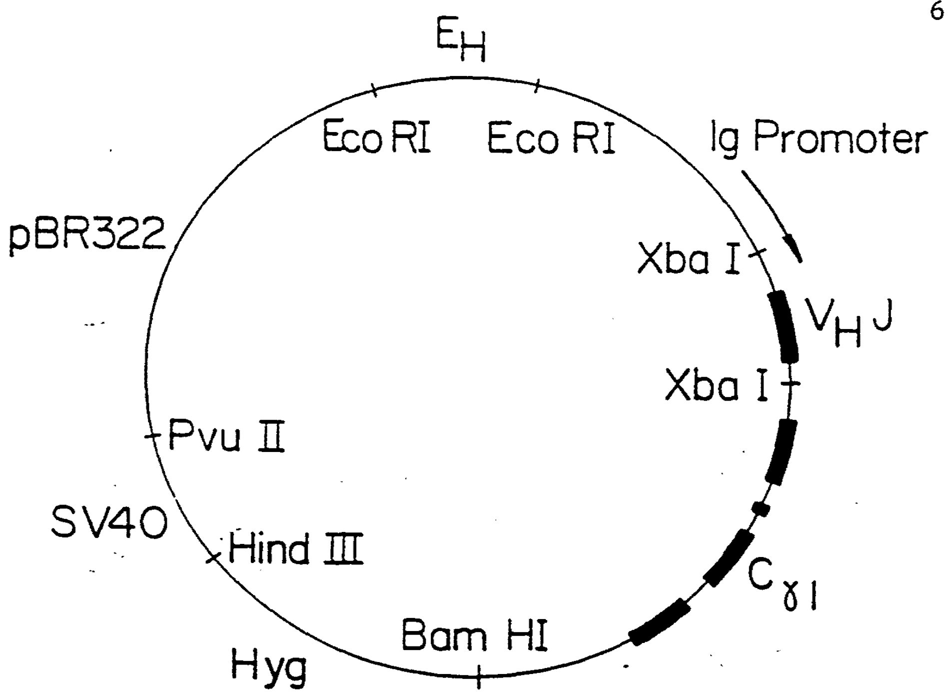


FIG._6.

SUBSTITUTE SHEET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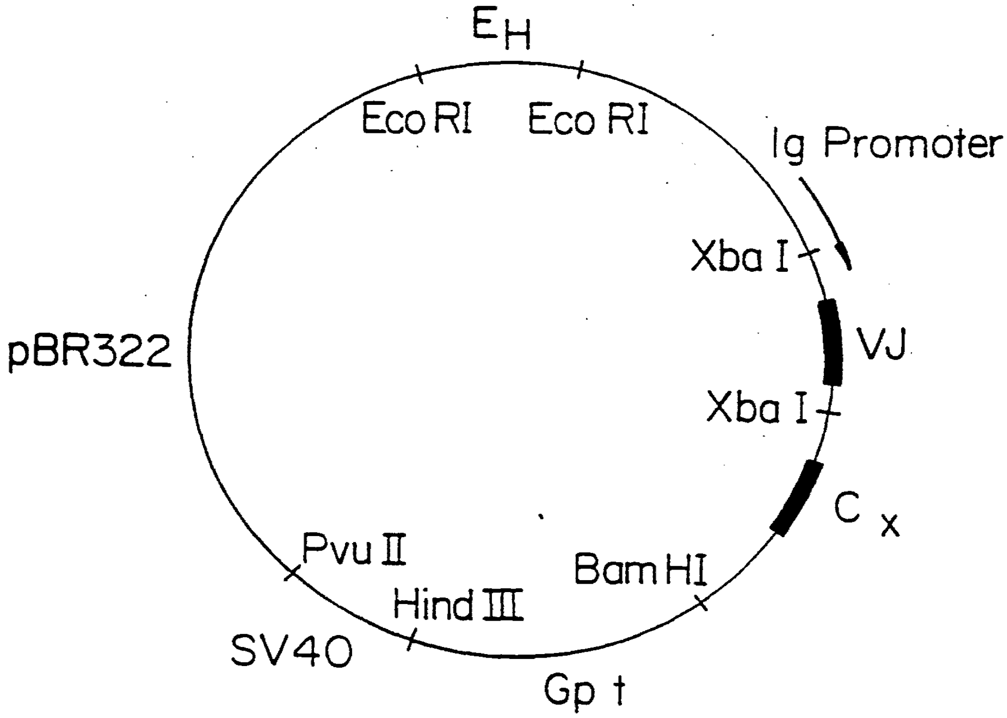
Eco RI
Eco RI
Ig Promoter
pBR322
Xba I
V_H J
Xba I
Pvu II
SV40
Hind III
Cγ1
Bam HI
Hyg

FIG._7.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Eco RI
Eco RI
Ig Promoter
Xba I
pBR322
VJ
Xba I
C x
Pvu II
Bam HI
Hind III
SV40
Gp t

FIG._8.

International Application No. PCT/US89/05857

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

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

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 [1], specifically:

3.☐ Claim numbers____________, because they are dependent claims 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:

See Attachment Sheet, (page 4).

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.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 4-90)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US89/05857

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

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

IPC (5) : C12P 21/00; C12N 5/10,7/01,15/00
U.S. Cl : 530/387; 435/69.1,240.1; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	530/387; 424/85; 435/69.1,172.3; 536/27; 435/240.1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y,P	US,A 4,816,567 (CABILLY ET AL) Issued 28 March 1989, See entire document.	1-22
X Y	EP,A 239,400 (WINTER) Issued 30 September 1987 See entire document.	18-22 1-17
X,P Y	WO,A 89/01783 (BODMER ET AL) Issued 09 March 1989, See entire document.	18-22 1-17
X Y	GB,A 2188941 (DIAMANTSTEIN ET AL) Issued 14 October 1987. See entire document.	1-4,7-8,14-15 5-6,9-13 & 16-22
Y	Science, Volume 238 Issued 20 November 1987 (VITETTA ET AL) "Redesigning nature's poisons to create anti-tumor reagents" , pp 1098-1104. See entire document.	1-22

(con't)

* Special categories of cited documents: [15]

"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

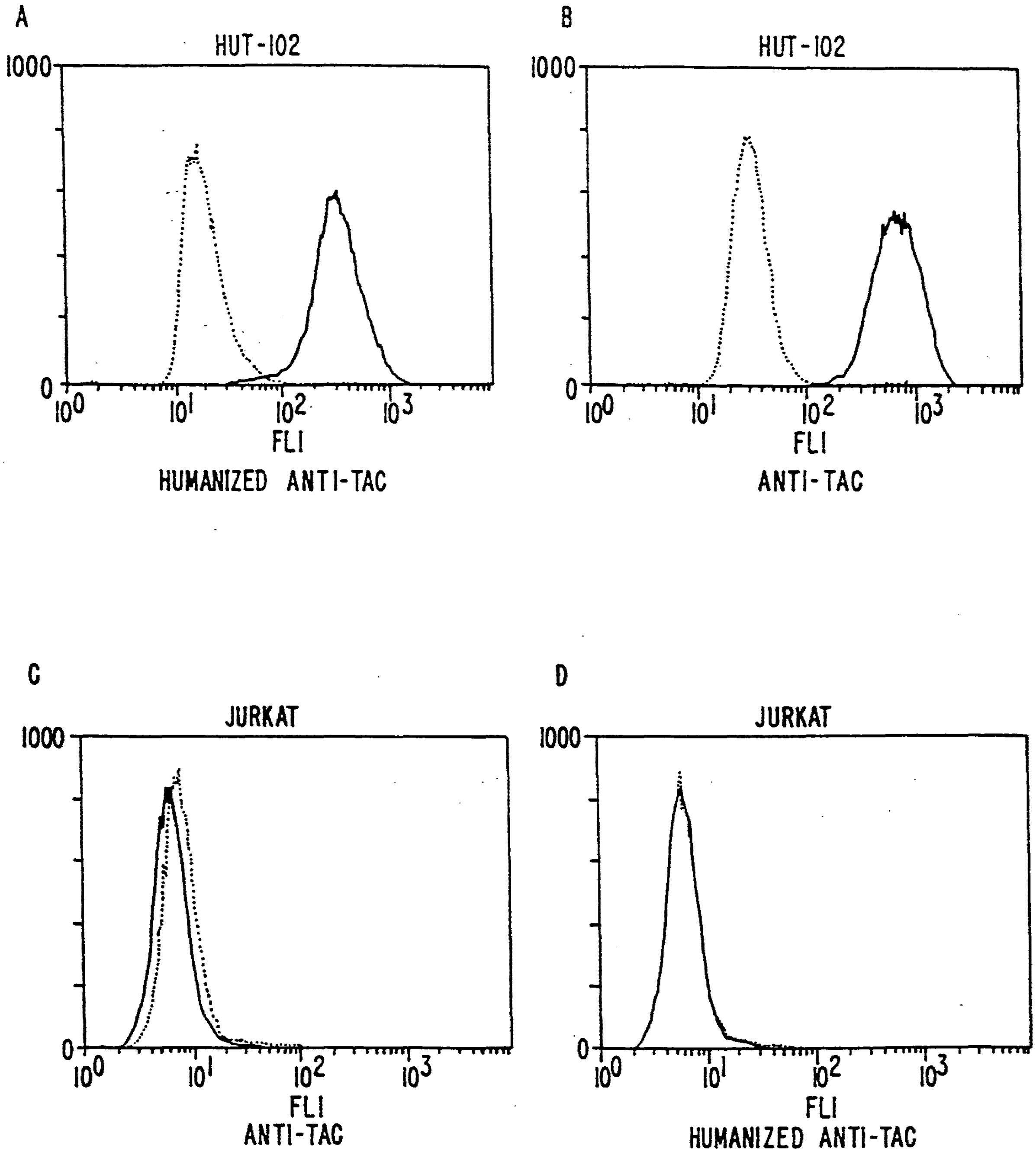
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2] 01 June 1990	Date of Mailing of this International Search Report [2] 02 JUL 1990
International Searching Authority [1] ISA/US	Signature of Authorized Officer [10] Michelle Marks

Form PCT/ISA/210 (second sheet) (May 1986)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10^3
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0
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10^1
10^2
10^3
FLI
ANTI-TAC
C
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ANTI-TAC
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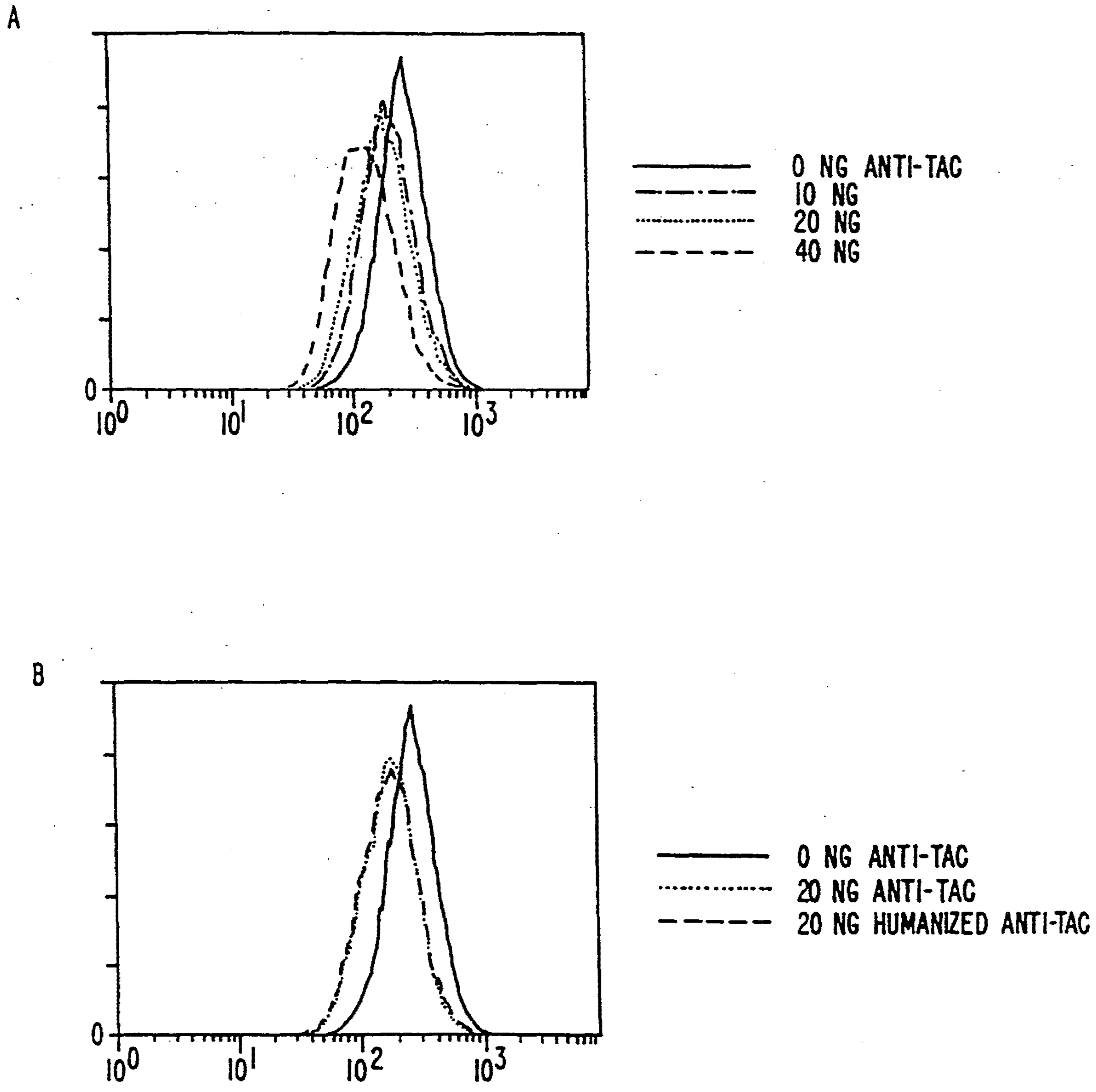
HUMANIZED ANTI-TAC

FIG._9.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10^2
10^3
0 NG ANTI-TAC
20 NG ANTI-TAC
20 NG HUMANIZED ANTI-TAC

FIG._10.

Form PTO 948 (Rev. 10-93) U.S. DEPARTMENT OF COMMERCE - Patent and Trademark Office Application No. 146 206

NOTICE OF DRAFTSPERSON'S PATENT DRAWING REVIEW

PTO Draftpersons review all originally filed drawings regardless of whether they are designated as formal or informal. Additionally, patent Examiners will review the drawings for compliance with the regulations. Direct telephone inquiries concerning this review to the Drawing Review Branch, 703-305-8404.

The drawings filed (insert date) 11/17/93, are
A. ___ not objected to by the Draftsperson under 37 CFR 1.84 or 1.152.
B. X objected to by the Draftsperson under 37 CFR 1.84 or 1.152 as indicated below. The Examiner will require submission of new, corrected drawings when necessary. Corrected drawings must be submitted according to the instructions on the back of this Notice.

1. DRAWINGS. 37 CFR 1.84(a): Acceptable categories of drawings: Black ink. Color.
- ___ Not black solid lines. Fig(s)______
- ___ Color drawings are not acceptable until petition is granted.

2. PHOTOGRAPHS. 37 CFR 1.84(b)
- ___ Photographs are not acceptable until petition is granted.

3. GRAPHIC FORMS. 37 CFR 1.84 (d)
- ___ Chemical or mathematical formula not labeled as separate figure. Fig(s)______
- ___ Group of waveforms not presented as a single figure, using common vertical axis with time extending along horizontal axis. Fig(s)______
- ___ Individuals waveform not identified with a separate letter designation adjacent to the vertical axis. Fig(s)______

4. TYPE OF PAPER. 37 CFR 1.84(e)
- ___ Paper not flexible, strong, white, smooth, nonshiny, and durable. Sheet(s)______
- ___ Erasures, alterations, overwritings, interlineations, cracks, creases, and folds not allowed. Sheet(s)______

5. SIZE OF PAPER. 37 CFR 1.84(f): Acceptable paper sizes:
21.6 cm. by 35.6 cm. (8 1/2 by 14 inches)
21.6 cm. by 33.1 cm. (8 1/2 by 13 inches)
21.6 cm. by 27.9 cm. (8 1/2 by 11 inches)
21.0 cm. by 29.7 cm. (DIN size A4)
- ___ All drawing sheets not the same size. Sheet(s)______
- ___ Drawing sheet not an acceptable size. Sheet(s)______

6. MARGINS. 37 CFR 1.84(g): Acceptable margins:

Paper size

	21.6 cm. X 35.6 cm. (8 1/2 X 14 inches)	21.6 cm X 33.1 cm. (8 1/2 X 13 inches)	21 cm. X 27.9 cm. (8 1/2 X 11 inches)	21 cm. X 29.7 cm. (DIN Size A4)
T	5.1 cm. (2")	2.5 cm. (1")	2.5 cm. (1")	2.5cm.
L	.64 cm. (1/4")	.64 cm. (1/4")	.64 cm. (1/4")	2.5 cm.
R	.64 cm. (1/4")	.64 cm. (1/4")	.64 cm. (1/4")	1.5 cm.
B	.64 cm. (1/4")	.64 cm. (1/4")	.64 cm. (1/4")	1.0 cm.

Margins do not conform to chart above.
Sheet(s)______
___ Top (T) X Left (L) ___ Right (R) ___ Bottom (B)

7. VIEWS. 37 CFR 1.84(h)
REMINDER: Specification may require revision to correspond to drawing changes.
- ___ All views not grouped together. Fig(s)______
- ___ Views connected by projection lines. Fig(s)______
- ___ Views contain center lines. Fig(s)______

Partial views. 37 CFR 1.84(h)(2)
- ___ Separate sheets not linked edge to edge. Fig(s)______
- ___ View and enlarged view not labeled separately. Fig(s)______
- ___ Long view relationship between different parts not clear and unambiguous. 37 CFR 1.84(h)(2)(ii) Fig(s)______

Sectional views. 37 CFR 1.84(h)(3)
- ___ Hatching not indicated for sectional portions of an object. Fig(s)______
- ___ Hatching of regularly spaced oblique parallel lines not spaced sufficiently. Fig(s)______
- ___ Hatching not at substantial angle to surrounding axes or principal lines. Fig(s)______
- ___ Cross section not drawn same as view with parts in cross section with regularly spaced parallel oblique strokes. Fig(s)______
- ___ Hatching of juxtaposed different elements not angled in a different way. Fig(s)______

Alternate position. 37 CFR 1.84(h)(4)
- ___ A separate view required for a moved position. Fig(s)______

Modified forms. 37 CFR 1.84(h)(5)
- ___ Modified forms of construction must be shown in separate views. Fig(s)______

8. ARRANGEMENT OF VIEWS. 37 CFR 1.84(i)
- ___ View placed upon another view or within outline of another. Fig(s)______
- ___ Words do not appear in a horizontal, left-to-right fashion when page is either upright or turned so that the top becomes the right side, except for graphs. Fig(s)______

9. SCALE. 37 CFR 1.84(k)
- ___ Scale not large enough to show mechanism without crowding when drawing is reduced in size to two-thirds in reproduction. Fig(s)______
- ___ Indication such as "actual size" or "scale 1/2" not permitted. Fig(s)______
- ___ Elements of same view not in proportion to each other. Fig(s)______

10. CHARACTER OF LINES, NUMBERS, & LETTERS. 37 CFR 1.84(l)
- ___ Lines, numbers & letters not uniformly thick and well defined, clean, durable, and black (except for color drawings). Fig(s)______

11. SHADING. 37 CFR 1.84(m)
- ___ Shading used for other than shape of spherical, cylindrical, and conical elements of an object, or for flat parts. Fig(s)______
- ___ Solid black shading areas not permitted. Fig(s)______

12. NUMBERS, LETTERS, & REFERENCE CHARACTERS. 37 CFR 1.84(p)
- ___ Numbers and reference characters not plain and legible. 37 CFR 1.84(p)(l) Fig(s)______
- ___ Numbers and reference characters used in conjuction with brackets, inverted commas, or enclosed within outlines. 37 CFR 1.84(p)(l) Fig(s)______
- ___ Numbers and reference characters not oriented in same direction as the view. 37 CFR 1.84(p)(l) Fig(s)______
- ___ English alphabet not used. 37 CFR 1.84(p)(2) Fig(s)______
- ___ Numbers, letters, and reference characters do not measure at least .32 cm. (1/8 inch) in height. 37 CFR(p)(3) Fig(s)______

13. LEAD LINES. 37 CFR 1.84(q)
- ___ Lead lines cross each other. Fig(s)______
- ___ Lead lines missing. Fig(s)______
- ___ Lead lines not as short as possible. Fig(s)______

14. NUMBERING OF SHEETS OF DRAWINGS. 37 CFR 1.84(t)
- X Number appears in top margin. Fig(s) 1/8
- ___ Number not larger than reference characters. Fig(s)______
- ___ Sheets not numbered consecutively, and in Arabic numerals, beginning with number 1. Sheet(s)______

15. NUMBER OF VIEWS. 37 CFR 1.84(u)
- ___ Views not numbered consecutively, and in Arabic numerals, beginning with number 1. Fig(s)______
- ___ View numbers not preceded by the abbreviation Fig. Fig(s)______
- ___ Single view contains a view number and the abbreviation Fig.
- ___ Numbers not larger than reference characters. Fig(s)______

16. CORRECTIONS. 37 CFR 1.84(w)
- ___ Corrections not durable and permanent. Fig(s)______

17. DESIGN DRAWING. 37 CFR 1.152
- ___ Surface shading shown not appropriate. Fig(s)______
- ___ Solid black shading not used for color contrast. Fig(s)______

ATTACHMENT TO PAPER NO. 12 REVIEWER______ DATE 5/11/94

PTO Copy

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37 APR 17 1995
PAT. & TRADEMARK OFF.

#13
4/26/95

PATENT DOCKET P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Carter and Presta

Serial No: 08/146,206

Filed: 17 November 1993

For: METHOD OF MAKING HUMANIZED ANTIBODIES

Group Art Unit: 1806 1802

Examiner: ADAMS, D.

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on

13 April 1995
(Date of Deposit)

Aida A. Miclat
Name of Depositing Party

Aida A. Miclat
Signature of Depositing Party

13 April 1995
Date of Signature

INFORMATION DISCLOSURE STATEMENT

Honorable Commissioner of Patents
and Trademarks
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 ($210) 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 $210.00 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.**

[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(b), 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.

A copy of a document pursuant to 37 C.F.R. § 10.9(b) is attached as proof of the authorization of the undersigned to prosecute the above-mentioned application. The original of this document is on file in the Office of Enrollment and Discipline.

Respectfully submitted,
GENENTECH, INC.

Date: April 13, 1995

By: WMLee
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

Sheet 1 of 4

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	
		Filing Date 17 Nov 1993	Group 1806

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37 APR 17 1995
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U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
TDS	1	4,816,567	28.03.89	Cabilly et al.	530	387.3	

FOREIGN PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
TDS	2	~~0 239 400~~	~~30.09.87~~	~~EPO~~				
	3	0 620 276	19.10.94	EPO				
	4	WO 89/01783	09.03.89	PCT				
	5	WO 89/06692	27.07.89	PCT				
	6	~~WO 90/07861~~	~~26.07.90~~	~~PCT~~				
	7	WO 91/09967	11.07.91	PCT				
	8	WO 92/22653	23.12.92	PCT				
	9	WO 93/02191	04.02.93	PCT				

OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, etc.)

TDS	10	Amzel and Poljak, "Three-dimensional structure of immunoglobulins" Ann. Rev. Biochem. 48:961-967 (1979)
	11	Bindon et al., "Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q" Journal of Experimental Medicine 168(1):127-142 (July 1988)
	12	Boulianne, G. L. et al., "Production of functional chimaeric mouse/human antibody" Nature 312(5995):643-646 (December 1984)
	13	Brown et al., "Anti-Tac-H, a humanized antibody to the interleukin 2 receptor, prolongs primate cardiac allograft survival" Proc. Natl. Acad. Sci. USA 88:2663-2667 (1991)
	14	Bruccoleri, "Structure of antibody hypervariable loops reproduced by a conformational search algorithm" Nature (erratum to article in Nature 335(6190):564-568 and) 336:266 (1988)
	15	Bruggemann, M. et al., "Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies" Journal of Experimental Medicine 166:1351-1361 (1987)
	16	Burgess et al., "Possible Dissociation of the Heparin-binding and Mitogenic Activities of Heparin-binding (Acidic Fibroblast) Growth Factor-1 from Its Receptor-binding Activities by Site-directed Mutagenesis of a Single Lysine Residue" Journal of Cell Biology 111:2129-2138 (1990)
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*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.

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Sheet 2 of 4

FORM PTO-1449 **LIST OF DISCLOSURES CITED BY APPLICANT** (Use several sheets if necessary)	U.S. Dept. of Commerce Patent and Trademark Office	Atty Docket No. P0709P1	Serial No. 08/146,206
		Applicant Carter and Presta	
		Filing Date 17 Nov 1993	Group ~~1806~~ 1816

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FORM PTO-1449 **LIST OF DISCLOSURES CITED BY APPLICANT** (Use several sheets if necessary)	U.S. Dept. of Commerce Patent and Trademark Office	Atty Docket No. P0709P1	Serial No. 08/146,206
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LIST OF DISCLOSURES CITED BY APPLICANT (Use several sheets if necessary)		Applicant Carter and Presta	DEC 20 1995
		Filing Date 17 Nov 1993	Group ~~1806~~ 1816

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Examiner	Date Considered 12/16/96

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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: 1806

Examiner: D. Adams

CERTIFICATE OF MAILING

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REQUEST FOR A CORRECTED FILING RECEIPT

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Sir:

Attached is a copy of the Official Filing Receipt received from the PTO in the above application for which issuance of a corrected filing receipt is respectfully requested. Please make the correction as follows: Under "CONTINUING DATA AS CLAIMED BY APPLICANT-", please delete "07/715,222 06/14/91 PAT D 335,559" and insert --07/715,272 06/14/91 ABD--.

The correction is not due to any error by applicant and no fee is believed to be due. However, in the event that the Patent Office determines that fees are due in connection with the filing of this document, we hereby authorize the Commissioner to charge such fees to our Deposit Account No. 07-0630.

A copy of a document pursuant to 37 C.F.R. § 10.9(b) is attached as proof of the authorization of the undersigned to prosecute the above-mentioned application. The original of this document is on file in the Office of Enrollment and Discipline.

Respectfully submitted,

GENENTECH, INC.

Date: April 10, 1995

By: WMLee
Wendy M. Lee

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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.

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APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTORNEY DOCKET NO.	DRWGS	TOT CL	IND CL
08/146,206	11/17/93	1806	$1,592.00	709P1	9	18	9

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Receipt is acknowledged of this patent application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please write to the Application Processing Division's Customer Correction Branch within 10 days of receipt. Please provide a copy of the Filing Receipt with the changes noted thereon.

Applicant(s) PAUL J. CARTER, SAN FRANCISCO, CA; LEONARD G. PRESTA, SAN FRANCISCO, CA.

CONTINUING DATA AS CLAIMED BY APPLICANT-
THIS APPLN IS A 371 OF PCT/US92/05126 06/15/92
WHICH IS A CIP OF ~~07/715,222 06/14/91 PAT D 335,559~~
07/715,272 06/14/91 ABD

FOREIGN/PCT APPLICATIONS-PCT PCT/US92/05126 06/15/92

TITLE
METHOD FOR MAKING HUMANIZED ANTIBODIES

PRELIMINARY CLASS: 530

PCT
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{5} : **C12N 15/13, C12P 21/08 C07K 13/00, C12N 5/10 G06F 15/00**	**A1**	**(11) International Publication Number:** **WO 92/22653** **(43) International Publication Date:** 23 December 1992 (23.12.92)

(21) International Application Number: PCT/US92/05126

(22) International Filing Date: 15 June 1992 (15.06.92)

(30) Priority data:
715,272 14 June 1991 (14.06.91) US

(60) Parent Application or Grant
(63) Related by Continuation
US 715,272 (CIP)
Filed on 14 June 1991 (14.06.91)

(71) Applicant *(for all designated States except US)*: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).

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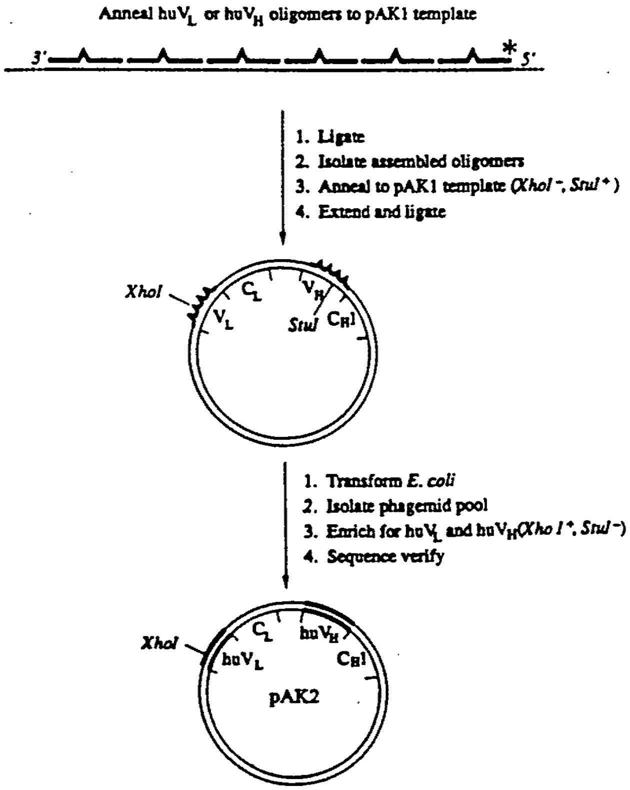
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR MAKING HUMANIZED ANTIBODIES

(57) Abstract

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



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⑪ Publication number: **0 239 400 A2**

⑫ EUROPEAN PATENT APPLICATION

㉑ Application number: 87302620.7

㉒ Date of filing: 26.03.87

(51) Int. Cl.⁴: C 12 N 15/00, C 07 K 15/06,
C 12 P 21/02

㉚ Priority: 27.03.86 GB 8607679

㊸ Date of publication of application: 30.09.87
Bulletin 87/40

(84) Designated Contracting States: AT BE CH DE ES FR GB
GR IT LI LU NL SE

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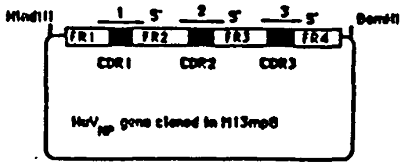
(54) Recombinant antibodies and methods for their production.

(57) An altered antibody is produced by replacing the complementarity determining regions (CDRs) of a variable region of an immunoglobulin (Ig) with the CDRs from an Ig of different specificity, using recombinant DNA techniques. The gene coding sequences for producing the altered antibody may be produced by site-directed mutagenesis using long oligonucleotides.



D1.3 CDR1 oligonucleotide
5' CTG,TCT,CAC,CCA,GTT,TAC,ACC,ATA,GCC,GCT,GAA,GGT,GCT
FR2 D1.3 CDR1 FR1

D1.3 CDR2 oligonucleotide
5' CAT,TGT,CAC,TCT,GGA,TTT,GAG,AGC,TGA,ATT,ATA,GTC,TGT,
FR3 D1.3 CDR2
GTT,TCC,ATC,ACC,CCA,AAT,CAT,TCC,AAT,CCA,CTC
D1.3 CDR2 FR2

D1.3 CDR3 oligonucleotide
5' GCC,TTG,ACC,CCA,GTA,GTC,AAG,CCT,ATA,ATC,TCT,CTC,TCT,
FR4 D1.3 CDR3
TGC,ACA,ATA
FR3

EP 0 239 400 A2

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Carter et al. P0709P1
SN: 08/146,206
Filed November 17, 1993

TITLE MODIFIED
see front page

RECOMBINANT DNA PRODUCT AND METHODS

The present invention relates to altered antibodies in which at least parts of the complementarity determining regions (CDRs) in the light or heavy chain variable domains of the antibody have been replaced by analogous parts of CDRs from an antibody of different specificity. The present invention also relates to methods for the production of such altered antibodies.

Natural antibodies, or immunoglobulins, comprise two heavy chains linked together by disulphide bonds and two light chains, one light chain being linked to each of the heavy chains by disulphide bonds. The general structure of an antibody of class IgG (i.e. an immunoglobulin (Ig) of class gamma (G)) is shown schematically in Figure 1 of the accompanying drawings.

Each heavy chain has at one end a variable domain followed by a number of constant domains. Each light chain has a variable domain at one end and a constant domain at its other end, the variable domain being aligned with the variable domain of the heavy chain and the constant domain being aligned with the first constant domain of the heavy chain. The constant domains in the light and heavy chains are not involved directly in binding the antibody to the antigen.

The variable domains of each pair of light and heavy chains form the antigen binding site. The domains on the light and heavy chains have the same general structure and each domain comprises four framework regions, whose sequences are relatively conserved, connected by three hypervariable or complementarity determining regions (CDRs) (see Kabat, E.A., Wu, T.T., Bilofsky, H., Reid-Miller, M. and Perry, H., in "Sequences of Proteins of Immunological Interest", US Dept. Health and Human Services 1983). The four framework regions largely adopt a β-sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs are held in close proximity by the framework regions and, with the CDRs from the other domain, contribute to the formation of the antigen binding site.

For a more detailed account of the structure of variable domains, reference may be made to: Poljak, R.J., Amzel, L.M., Avey, H.P., Chen, B.L., Phizackerly, R.P. and Saul, F., PNAS USA, 70, 3305-3310, 1973; Segal, D.M., Padlan, E.A., Cohen, G.H., Rudikoff, S., Potter, M. and Davies, D.R., PNAS USA, 71, 4298-4302, 1974; and Marquart, M., Deisenhofer, J., Huber, R. and Palm, W., J. Mol. Biol., 141, 369-391, 1980.

In recent years advances in molecular biology based on recombinant DNA techniques have provided processes for the production of a wide range of heterologous polypeptides by transformation of host cells with heterologous DNA sequences which code for the production of the desired products.

EP-A-0 088 994 (Schering Corporation) proposes the construction of recombinant DNA vectors comprising a ds DNA sequence which codes for a variable domain of a light or a heavy chain of an Ig specific for a predetermined ligand. The ds DNA sequence is provided with initiation and termination codons at its 5'- and 3'-termini respectively, but lacks any nucleotides coding for amino acids superfluous to the variable domain. The ds DNA sequence is used to transform bacterial cells. The application does not contemplate variations in the sequence of the variable domain.

EP-A-1 102 634 (Takeda Chemical Industries Limited) describes the cloning and expression in bacterial host organisms of genes coding for the whole or a part of human IgE heavy chain polypeptide, but does not contemplate variations in the sequence of the polypeptide.

EP-A-0 125 023 (Genentech Inc.) proposes the use of recombinant DNA techniques in bacterial cells to produce Ig's which are analogous to those normally found in vertebrate systems and to take advantage of the gene modification techniques proposed therein to construct chimeric Igs or other modified forms of Ig.

The term 'chimeric antibody' is used to describe a protein comprising at least the antigen binding portion of an immunoglobulin molecule (Ig) attached by peptide linkage to at least part of another protein.

It is believed that the proposals set out in the above Genentech application did not lead to the expression of any significant quantities of Ig polypeptide chains, nor to the production of Ig activity, nor to the secretion and assembly of the chains into the desired chimeric Igs.

The production of monoclonal antibodies was first disclosed by Kohler and Milstein (Kohler, G. and Milstein, C., Nature, 256, 495-497, 1975). Such monoclonal antibodies have found widespread use not only as diagnostic reagents (see, for example, 'Immunology for the 80s, Eds. Voller, A., Bartlett, A., and Bidwell, D., MTP Press, Lancaster, 1981) but also in therapy (see, for example, Ritz, J. and Schlossman, S.F., Blood, 59, 1-11, 1982).

The recent emergence of techniques allowing the stable introduction of Ig gene DNA into myeloma cells (see, for example, Oi, V.T., Morrison, S.L., Herzenberg, L.A. and Berg, P., PNAS USA, 80, 825-829, 1983; Neuberger, M.S., EMBO J., 2, 1373-1378, 1983; and Ochi, T., Hawley, R.G., Hawley, T., Schulman, M.J., Traunecker, A., Kohler, G. and Hozumi, N., PNAS USA, 80, 6351-6355, 1983), has opened up the possibility of using in vitro mutagenesis and DNA transfection to construct recombinant Igs possessing novel properties.

However, it is known that the function of an Ig molecule is dependent on its three dimensional structure, which in turn is dependent on its primary amino acid sequence. Thus, changing the amino acid sequence of an Ig may adversely affect its activity. Moreover, a change in the DNA sequence coding for the Ig may affect the ability of the cell containing the DNA sequence to express, secrete or assemble the Ig.

It is therefore not at all clear that it will be possible to produce functional altered antibodies by recombinant DNA techniques.

However, colleagues of the present Inventor have devised a process whereby chimeric antibodies in which both parts of the protein are functional can be secreted. The process, which is disclosed in International Patent Application No. PCT/GB85/00392 (Neuberger et al. and Celltech Limited), comprises:

a) preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence comprising a first part which encodes at least the variable domain of the heavy or light chain of an Ig molecule and a second part which encodes at least part of a second protein;

b) if necessary, preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary light or heavy chain respectively of an Ig molecule;

c) transforming an immortalised mammalian cell line with the or both prepared vectors; and

d) culturing said transformed cell line to produce a chimeric antibody.

The second part of the DNA sequence may encode:

i) at least part, for instance the constant domain of a heavy chain, of an Ig molecule of different species, class or subclass;

ii) at least the active portion or all of an enzyme;

iii) a protein having a known binding specificity;

iv) a protein expressed by a known gene but whose sequence, function or antigenicity is not known; or

v) a protein toxin, such as ricin.

The above Neuberger application only shows the production of chimeric antibodies in which complete variable domains are coded for by the first part of the DNA sequence. It does not show any chimeric antibodies in which the sequence of the variable domain has been altered.

The present invention, in a first aspect, provides an altered antibody in which at least parts of the CDRs in the light or heavy chain variable domains have been replaced by analogous parts of CDRs from an antibody of different specificity

The determination as to what constitutes a CDR and what constitutes a framework region was made on the basis of the amino-acid sequences of a number of Igs. However, from the three dimensional structure of a number of Igs it is apparent that the antigen binding site of an Ig variable domain comprises three looped regions supported on sheet-like structures. The loop regions do not correspond exactly to the CDRs, although in general there is considerable overlap.

Moreover, not all of the amino-acid residues in the loop regions are solvent accessible and in one case, amino-acid residues in the framework regions are involved in antigen binding.(Amit, A.G., Mariuzza, R.A., Phillips, S.E.V. and Poljak, R.J., Science, 233, 747-753, 1986).

It is also known that the variable regions of the two parts of an antigen binding site are held in the correct orientation by inter-chain non-covalent interactions. These may involve amino-acid residues within the CDRs.

Thus, in order to transfer the antigen binding capacity of one variable domain to another, it may not be necessary to replace all of the CDRs with the complete CDRs from the donor variable region. It may be necessary only to transfer those residues which are accessible from the antigen binding site, and this may involve transferring framework region residues as well as CDR residues.

It may also be necessary to ensure that residues essential for inter-chain interactions are preserved in the acceptor variable domain.

Within a domain, the packing together and orientation of the two disulphide bonded β-sheets (and therefore the ends of the CDR loops) are relatively conserved. However, small shifts in packing and orientation of these β-sheets do occur

(Lesk, A.M. and Chothia, C., J. Mol. Biol., 160, 325-342, 1982). However, the packing together and orientation of heavy and light chain variable domains is relatively conserved (Chothia, C., Novotny, J., Bruccoleri, R. and Karplus, M., J. Mol. Biol., 186, 651-653, 1985). These points will need to be borne in mind when constructing a new antigen biding site so as to ensure that packing and orientation are not altered to the deteriment of antigen binding capacity.

It is thus clear that merely by replacing one or more CDRs with complementary CDRs may not always result in a functional altered antibody. However, given the explanations set out above, it will be well within the competence of the man skilled in the art, either by carrying out routine experimentation or by trial and error testing to obtain a functional altered antibody.

Preferably, the variable domains in both the heavy and light chains have been altered by at least partial CDR replacement and, if necessary, by partial framework region replacement and sequence changing. Although the CDRs may be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of different class and preferably from an antibody from a different species.

Thus, it is envisaged, for instance, that the CDRs from a mouse antibody could be grafted onto the framework regions of a human antibody. This arrangement will be of particular use in the therapeutic use of monoclonal antibodies.

At present, when a mouse monoclonal antibody or even a chimeric antibody comprising a complete mouse variable domain is injected into a human, the human body's immune system recognises the mouse variable domain as foreign and produces an immune response thereto. Thus, on subsequent injections of the mouse antibody or chimeric antibody into the human, its effectiveness is considerably reduced by the action of the body's immune system against the foreign antibody. In the altered antibody of the present invention, only the CDRs of the antibody will be foreign to the body, and this should minimise side effects if used for human therapy. Although, for example, human and mouse framework regions have characteristic sequences, there seem to be no characteristic features which distinguish human from mouse CDRs. Thus, an antibody comprised of mouse CDRs in a human framework may well be no more foreign to the body than a genuine human antibody.

Even with the altered antibodies of the present invention, there is likely to be an anti-idiotypic response by the recipient of the altered antibody. This response is directed to the antibody binding region of the altered antibody, It is believed that at least some anti-idiotype antibodies are directed at sites bridging the CDRs and the framework regions. It would therefore be possible to provide a panel of antibodies having the same partial or complete CDR replacements but on a series of different framework regions. Thus, once a first altered antibody became therapeutically ineffective, due to an anti-idiotype response, a second altered antibody from the series could be used, and so on, to overcome the effect of the anti-idiotype response. Thus, the useful life of the antigen-binding capacity of the altered antibodies could be extended.

Preferably, the altered antibody has the structure of a natural antibody or a fragment thereof. Thus, the altered antibody may comprise a complete antibody, an $(Fab')_2$ fragment, an Fab fragment, a light chain dimer or a heavy chain dimer. Alternatively, the altered antibody may be a chimeric antibody of the type described in the Neuberger application referred to above. The production of such an altered chimeric antibody can be carried out using the methods described below used in conjunction with the methods described in the Neuberger application.

The present invention, in a second aspect, comprises a method for producing such an altered antibody comprising:

a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least a variable domain of an Ig heavy or light chain, the variable domain comprising framework regions from a first antibody and CDRs comprising at least parts of the CDRs from a second antibody of different specificity;

b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary Ig light or heavy chain respectively;

c) transforming a cell line with the first or both prepared vectors; and

d) culturing said transformed cell line to produce said altered antibody.

The present invention also includes vectors used to transform the cell line, vectors used in producing the transforming vectors, cell lines transformed with the transforming vectors, cell lines tranformed with preparative vectors, and methods for their production.

Preferably, the cell line which is transformed to produce the altered antibody is an immortalised mammalian cell line, which is advantageously of lymphoid origin, such as a myeloma, hybridoma, trioma or quadroma cell line. The cell line may also comprise a normal lymphoid cell, such as a B-cell, which has been immortalised by transformation with a virus, such as the Epstein-Barr virus. Most preferably, the immortalised cell line is a myeloma cell line or a derivative thereof.

Although the cell line used to produce the altered antibody is preferably a mammalian cell line, any other suitable cell line, such as a bacterial cell line or a yeast cell line, may alternatively be used. In particular, it is envisaged that E. Coli derived bacterial strains could be used.

It is known that some immortalised lymphoid cell lines, such as myeloma cell lines, in their normal state secrete isolated Ig light or heavy chains. If such a cell line is transformed with the vector prepared in step a) of the process of the invention, it will not be necessary to carry out step b) of the process, provided that the normally secreted chain is complementary to the variable domain of the Ig chain encoded by the vector prepared in step a).

However, where the immortalised cell line does not secrete or does not secrete a complementary chain, it will be necessary to carry out step b). This step may be carried out by further manipulating the vector produced in step a) so that this vector encodes not only the variable domain of an altered antibody light or heavy chain, but also the complementary variable domain.

Alternatively, step b) is carried out by preparing a second vector which is used to transform the immortalised cell line. This alternative leads to easier construct preparation, but may be less preferred than the first alternative in that it may not lead to as efficient production of antibody.

The techniques by which such vectors can be produced and used to transform the immortalised cell lines are well known in the art, and do not form any part of the invention.

In the case where the immortalised cell line secretes a complementary light or heavy chain, the transformed cell line may be produced for example by transforming a suitable bacterial cell with the vector and then fusing the bacterial cell with the immortalised cell line by spheroplast fusion. Alternatively, the DNA may be directly introduced into the immortalised cell line by electroporation.

The DNA sequence encoding the altered variable domain may be prepared by oligonucleotide synthesis. This requires that at least the framework region sequence of the acceptor antibody and at least the CDRs sequences of the donor antibody are known or can be readily determined. Although determining these sequences, the synthesis of the DNA from oligonucleotides and the preparation of suitable vectors is to some extent laborious, it involves the use of known techniques which can readily be carried out by a person skilled in the art in light of the teaching given here.

If it was desired to repeat this strategy to insert a different antigen binding site, it would only require the synthesis of oligonucleotides encoding the CDRs, as the framework oligonucleotides can be re-used.

A convenient variant of this technique would involve making a symthetic gene lacking the CDRs in which the four framework regions are fused together with suitable restriction sites at the junctions. Double stranded synthetic CDR cassettes with sticky ends could then be ligated at the junctions of the framework regions. A protocol for achieving this variant is shown diagrammatically in Figure 6 of the accompanying drawings.

Alternatively, the DNA sequence encoding the altered variable domain may be prepared by primer directed oligonucleotide site-directed mutagenesis. This technique in essence involves hybridising an oligonucleotide coding for a desired mutation with a single strand of DNA containing the region to be mutated and using the single strand as a template for extension of the oligonulcleotide to produce a strand containing the mutation. This technique, in various forms, is described by : Zoller, M.J. and Smith, M., Nuc. Acids Res., 10, 6487-6500, 1982; Norris, K., Norris F., Christiansen, L. and Fiil, N., Nuc. Acids Res., 11, 5103-5112, 1983; Zoller, M.J. and Smith, M., DNA, 3, 479-488 (1984); Kramer, W., Schughart, K. and Fritz, W.-J., Nuc. Acids Res., 10, 6475-6485, 1982.

For various reasons, this technique in its simplest form does not always produce a high frequency of mutation. An improved technique for introducing both single and multiple mutations in an M13 based vector, has been described by Carter et al. (Carter, P., Bedouelle H. and Winter, G., Nuc. Acids Res., 13, 4431-4443, 1985)

Using a long oligonucleotide, it has proved possible to introduce many changes simultaneously (as in Carter et al., loc. cit.) and thus single oligonucleotides, each encoding a CDR, can be used to introduce the three CDRs from a second antibody into the framework regions of a first antibody. Not only is this technique less laborious than total gene synthesis, but it represents a particularly convenient way of expressing a variable domain of required specificity, as it can be simpler than tailoring an entire V_H domain for insertion into an expression plasmid.

The oligonucleotides used for site-directed mutagenesis may be prepared by oligonucleotide synthesis or may be isolated from DNA coding for the variable domain of the second antibody by use of suitable restriction enzymes. Such long oligonucleotides will generally be at least 30 bases long and may be up to or over 80 bases in length.

The techniques set out above may also be used, where necessary, to produce the vector of part (b) of the process.

The method of the present invention is envisaged as being of particular use in "humanising" non-human monoclonal antibodies. Thus, for instance, a mouse monoclonal antibody against a particular human cancer cell may be produced by techniques well known in the art. The CDRs from the mouse monoclonal antibody may then be partially or totally grafted into the framework regions of a human monoclonal antibody, which is then produced in quantity by a suitable cell line. The product is thus a specifically targetted, essentially human antibody which will recognise the cancer cells, but will not itself be recognised to any significant degree, by a human's immune system, until the anti-idiotype response eventually becomes apparent. Thus, the method and product of the present invention will be of particular use in the clinical environment.

The present invention is now described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 is a schematic diagram showing the structure of an IgG molecule;

Figure 2 shows the amino acid sequence of the V_H domain of NEWM in comparison with the V_H domain of the BI-8 antibody;

Figure 3 shows the amino acid and nucleotide sequence of the HuV_{NP} gene;

Figure 4 shows a comparison of the results for HuV_{NP}-IgE and MoV_{NP}-IgE in binding inhibition assays;

Figure 5 shows the structure of three oligonucleotides used for site directed mutagenesis;

Figure 6 shows a protocol for the construction of CDR replacements by insertion of CDR cassettes into a vector containing four framework regions fused together;

Figure 7 shows the sequence of the variable domain of antibody D1.3 and the gene coding therefor; and

Figure 8 shows a protocol for the cloning of the D1.3 variable domain gene.

EXAMPLE 1

This example shows the production of an altered antibody in which the variable domain of the heavy chains comprises the framework regions of a human heavy chain and the CDRs from a mouse heavy chain.

The framework regions were derived from the human myeloma heavy chain NEWM, the crystallographic structure of which is known (see Poljak et al., loc. cit. and Reth, M., Hammerling, G.J. and Rajewsky, K., EMBO J., 1, 629-634, 1982.)

The CDRs were derived from the mouse monoclonal antibody B1-8 (see Reth et al., loc. cit.), which binds the hapten NP-cap (4-hydroxy-3-nitrophenyl acetyl-caproic acid: $K_{NP-CAP}=1.2\ \mu M$).

A gene encoding a variable domain HuV_{NP}, comprising the B1-8 CDRs and the NEWM framework regions, was constructed by gene synthesis as follows.

The amino acid sequence of the V_H domain of NEWM is shown in Figure 2, wherein it is compared to the amino acid sequence of the V_H domain of the B1-8 antibody. The sequence is divided into framework regions and CDRs according to Kabat et al. (loc. cit.). Conserved residues are marked with a line.

The amino acid and nucleotide sequence of the HuV_{NP} gene, in which the CDRs from the B1-8 antibody alternate with the framework regions of the NEWM antibody, is shown in Figure 3. The HuV_{NP} gene was derived by replacing sections of the MoV_{NP} gene in the vector $pSV-V_{NP}$ (see Neuberger, M.S., Williams, G.T., Mitchell, E.B., Jouhal, S., Flanagan, J.G. and Rabbitts, T.H., Nature, 314, 268-270, 1985) by a synthetic fragment encoding the HuV_{NP} domain. Thus the 5' and 3' non-coding sequences, the leader sequence, the L-V intron, five N-terminal and four

C-terminal amino acids are from the MoV_{NP} gene and the rest of the coding sequence is from the synthetic HuV_{NP} fragment.

The oligonucleotides from which the HuV_{NP} fragment was assembled are aligned below the corresponding portion of the HuV_{NP} gene. For convenience in cloning, the ends of oligonucleotides 25 and 26b form a Hind II site followed by a Hind III site, and the sequences of the 25/26b oligonucleotides therefore differ from the HuV_{NP} gene.

The HuV_{NP} synthetic fragment was built as a PstI-Hind III fragment. The nucleotide sequence was derived from the protein sequence using the computer programme ANALYSEQ (Staden, R., Nuc. Acids. Res., 12, 521-538, 1984) with optimal codon usage taken from the sequences of mouse constant domain genes. The oligonucleotides (1 to 26b, 28 in total) vary in size from 14 to 59 residues and were made on a Biosearch SAM or an Applied Biosystems machine, and purified on 8M-urea polyacrylamide gels (see Sanger, F. and Coulson, A., FEBS Lett., 87, 107-110, 1978).

The oligonucleotides were assembled in eight single stranded blocks (A-D) containing oligonucleotides

[1,3,5,7] (Block A), [2,4,6,8] (block A'), [9,11,13a,13b] (Block B), [10a, 10b,12/14] (block B'), [15, 17] (block C), [16,18] (block C'), [19, 21, 23, 25] (block D) and [20, 22/24, 26a, 26b) (block D').

In a typical assembly, for example of block A, 50 pmole of oligonucleotides 1,3,5 and 7 were phosphorylated at the 5' end with T4 polynucleotide kinase and mixed together with 5 pmole of the terminal oligonucleotide [1] which had been phosphorylated with 5 µCi [γ-^{32}P] ATP (Amersham 3000 Ci/mmole). These oligonucleotides were annealed by heating to 80°C and cooling over 30 minutes to room temperature, with unkinased oligonucleotides 2, 4 and 6 as splints, in 150 µl of 50 mM Tris.Cl, pH 7.5, 10 mM $MgCl_2$. For the ligation, ATP (1 mM) and DTT (10mM) were added with 50 U T4 DNA ligase (Anglian Biotechnology Ltd.) and incubated for 30 minutes at room temperature. EDTA was added to 10 mM, the sample was extracted with phenol, precipitated from ethanol, dissolved in 20 µl water and boiled for 1 minute with an equal volume of formamide dyes. The sample was loaded onto and run on a 0.3 mm 8M-urea 10% polyacrylamide gel. A band of the expected size was detected by autoradiography and eluted by soaking.

Two full length single strands were assembled from blocks A to D and A' to D' using splint oligonucleotides. Thus blocks A to D were annealed and ligated in 30 µl as set out in the previous paragraph using 100 pmole of oligonucleotides 10a, 16 and 20 as splints. Blocks A' to D' were ligated using oligonucleotides 7, 13b and 17 as splints.

After phenol/ether extraction, block A-D was annealed with block A'-D', small amounts were cloned in the vector M13mp18 (Yanish-Perron, C., Vieira, J. and Messing, J., Gene, 33, 103-119, 1985) cut with PstI and Hind III, and the gene sequenced by the dideoxy technique (Sanger, F., Nicklen, S. and Coulson, A.R., PNAS USA, <u>74</u>, 5463-5467, 1979).

The MoV$_{NP}$ gene was transferred as a Hind III - BamHI fragment from the vector pSV-V$_{NP}$ (Neuberger et al., loc. cit.) to the vector M13mp8 (Messing, J. and Vieira, J., Gene, <u>19</u>, 269-276, 1982). To facilitate the replacement of MoV$_{NP}$ coding sequences by the synthetic HuV$_{NP}$ fragment, three Hind II sites were removed from the 5' non-coding sequence by site directed mutagenesis, and a new Hind II site was subsequently introduced near the end of the fourth framework region (FR4 in Figure 2). By cutting the vector with PstI and Hind II, most of the V$_{NP}$ fragment can be inserted as a PstI-Hind II fragment. The sequence at the Hind II site was corrected to NEWM FR4 by site directed mutagenesis.

The Hind III - Bam HI fragment, now carrying the HuV$_{NP}$ gene, was excised from M13 and cloned back into pSV-V$_{NP}$ to replace the MoV$_{NP}$ gene and produce a vector pSV-HuV$_{NP}$. Finally, the genes for the heavy chain constant domains of human Ig E (Flanagan, J.G. and Rabbitts, T.H., EMBO J., <u>1</u>, 655-660, 1982) were introduced as a Bam HI fragment to give the vector pSV-HuV$_{NP}$. HE. This was transfected into the myeloma line J558 L by spheroplast fusion.

The sequence of the HuV$_{NP}$ gene in pSV-HuV$_{NP}$. HE was checked by recloning the Hind III-Bam HI fragment back into M13mp8 (Messing et al., loc. cit.). J558L myeloma cells secrete lambda 1 light chains which have been shown to associate with heavy chains containing the MoV$_{NP}$ variable domain to create a binding site for NP-cap or the related hapten NIP-Cap (3-iodo-4-hydroxy-5-nitrophenylacetyl-caproic acid) (Reth, M., Hammerling, G.J. and Rajewsky, K., Eur. J. Immunol., 8, 393-400, 1978).

As the plasmid pSV-HuV_{NP}.HE contains the *gpt* marker, stably transfected myeloma cells could be selected in a medium containing mycophenolic acid. Transfectants secreted an antibody (HuV_{NP}-IgE) with heavy chains comprising a HuV_{NP} variable domain (i.e. a "humanised" mouse variable region) and human γ constant domains, and lambda 1 light chains from the J558L myeloma cells.

The culture supernatants of several gpt^{+} clones were assayed by radioimmunoassay and found to contain NIP-cap binding antibody. The antibody secreted by one such clone was purified from culture supernatant by affinity chromatography on NIP-cap Sepharose (Sepharose is a registered trade mark). A polyacrylamide - SDS gel indicated that the protein was indistinguishable from the chimeric antibody MoV_{NP}-IgE (Neuberger et al., loc. cit.).

The HuV_{NP}-IgE antibody competes effectively with the MoV_{NP}-IgE for binding to both anti-human-IgE and to NIP-cap coupled to bovine serum albumin.

Various concentrations of HuV_{NP}-IgE and MoV_{NP}-IgE were used to compete the binding of radiolabelled MoV_{NP}-IgE to polyvinyl microtitre plates coated with (a) Sheep anti-human-IgE antiserum (Seward Laboratories); (b) NIP-cap-bovine serum albumin; (c) Ac38 anti-idiotypic antibody; (d) Ac 146 anti-idiotypic antibody; and (e) rabbit anti-MoV_{NP}

antiserum. Binding was also carried out in the presence of MoV_{NP}-IgM antibody (Neuberger, M.S., Williams, G.T. and Fox, R.O., Nature, 312, 604-608, 1984) or of JW5/1/2 which is an IgM antibody differing from the MoV_{NP}-IgM antibody at 13 residues mainly located in the V_H CDR2 region.

The results of the binding assays are shown in Figure 4, wherein black circles represent HuV_{NP}, white circles MoV_{NP}, black squares MoV_{NP}-IgM and white squares JW5/1/2. Binding is given relative to the binding in the absence of the inhibitor.

The affinities of HuV_{NP}-IgE for NP-cap and NIP-cap were then measured directly using the fluorescence quench technique and compared to those for MoVNP-IgE, using excitation at 295 nm and observing emission at 340 nm (Eisen, H.N., Methods Med. Res., 10, 115-121,1964).

Antibody solutions were diluted to 100 nM in phosphate buffered saline, filtered (0.45 µm pore cellulose acetate) and titrated with NP-cap in the range 0.2 to 20 µM. As a control, mouse DI-3 antibody (Mariuzza, R.A., Jankovic, D.L., Bulot, G., Amit, A.G., Saludjian, P., Le Guern, A., Mazie, J.C. and Poljak, R.J., J. Mol. Biol., 170, 1055-1058, 1983), which does not bind hapten, was titrated in parallel.

Decrease in the ratio of the fluorescence of HuV_{NP}-IgE or HuV_{NP}-IgE to the fluorescence of the D1-3 antibody was taken to be proportional to NP-cap occupancy of the antigen binding sites. The maximum quench was about 40% for both antibodies, and hapten dissociation constants were determined from least-squares fits of triplicate data sets to a hyperbola.

For NIP-cap, hapten concentration varied from 10 to 300 nM, and about 50% quenching of fluorescence was observed at saturation. Since the antibody concentrations were comparable to the value of the dissociation constants, data were fitted by least squares to an equation describing tight binding inhibition (Segal, I.H., in "Enzyme Kinetics", 73-74, Wiley, New York, 1975).

The binding constants obtained from these data for these antibodies are shown in Table 1 below.

Table 1

	$K_{NP\text{-}cap}$	$K_{NIP\text{-}cap}$
MoV_{NP}-IgE	1.2 μM	0.02 μM
HuV_{NP}-IgE	1.9 μM	0.07 μM

These results show that the affinities of these antibodies are similar and that the change in affinity is less than would be expected for the loss of a hydrogen bond or a van der Waals contact point at the active site of an enzyme.

Thus, it has been shown that it is possible to produce an antibody specific for an artificial small hapten, comprising a variable domain having human framework regions and mouse CDRs, without any significant loss of antigen binding capacity.

As shown in Figure 4(d), the HuV_{NP}-IgE antibody has lost the MoV_{NP} idiotypic determinant recognised by the antibody Ac146. Furthermore, HuV_{NP}-IgE also binds the Ac38 antibody less well (Figure 4(c)), and it is therefore not surprising that HuV_{NP}-IgE has lost many of the determinants recognised by the polyclonal rabbit anti-idiotypic antiserum (Figure 4(e)).

It can thus be seen that, although the HuV_{NP}-IgE antibody has acquired substantially all the antigen binding capacity of the mouse CDRs, it has not acquired any substantial proportion of the mouse antibody's antigenicity.

The results of Figures 4(d) and 4(e) carry a further practical implication. The mouse (or human) CDRs could be transferred from one set of human frameworks (antibody 1) to another (antibody 2). In therapy, anti-idiotypic antibodies generated in response to antibody 1 might well bind poorly to antibody 2. Thus, as the anti-idiotypic response starts to neutralise antibody 1 treatment could be continued with antibody 2, and the CDRs of a desired specificity used more than once.

For instance, the oligonucleotides encoding the CDRs may be used again, but with a set of oligonucleotides encoding a different set of framework regions.

The above work has shown that antigen binding characteristics can be transferred from one framework to another without loss of activity, so long as the original antibody is specific for a small hapten.

It is known that small haptens generally fit into an antigen binding cleft. However, this may not be true for natural antigens, for instance antigens comprising an epitopic site on a protein or polysaccharide. For such antigens, the antibody may lack a cleft (it may only have a shallow concavity), and surface amino acid residues may play a significant role in antigen binding. It is therefore not readily apparent that the work on artificial antigens shows conclusively that CDR replacement could be used to transfer natural antigen binding properties.

Therefore work was carried out to see if CDR replacement could be used for this purpose. This work also involved using primer-directed, oligonucleotide site-directed mutagenesis using three synthetic oligonucleotides coding for each of the mouse CDRs and the flanking parts of framework regions to produce a variable domain gene similar to the HuV_{NP} gene.

EXAMPLE 2

The three dimensional structure of a complex of lysozyme and the antilysozyme antibody D1.3 (Amit et al., loc. cit.) was solved by X-ray crystallography. There is a large surface of interaction between the antibody and antigen. The antibody has two heavy chains of the mouse IgG1 class (H) and two Kappa light chains (K), and is denoted below as H_2K_2.

The DNA sequence of the heavy chain variable region was determined by making cDNA from the mRNA of the D1.3 hybridoma cells, and cloning into plasmid and M13 vectors. The sequence is shown in Figure 7, in which the boxed residues comprise the three CDRs and the asterisks mark residues which contact lysozyme.

Three synthetic oligonucleotides were then designed to introduce the D1.3 V_HCDRs in place of the V_HCDRs of the HuV_{NP} gene. The Hu_{NP} gene has been cloned into M13mp8 as a BamHI-Hind III fragment, as described above. Each oligonucleotide has 12 nucleotides at the 5' end and 12 nucleotides at the 3' end which are complementary to the appropriate HuV_{NP} framework regions. The central portion of each oligonucleotide encodes either CDR1, CDR2, or CDR3 of the D1.3 antibody, as shown in Figure 5, to which reference is now made. It can be seen from this Figure that these oligonucleotides are 39, 72 and 48 nucleotides long respectively.

10 pmole of D1.3 CDR1 primer was phosphorylated at the 5' end and annealed to 1μg of the M13-HuV_{NP} template and extended with the Klenow fragment of DNA polymerase in the presence of T4 DNA ligase. After an oligonucleotide extension at 15°C, the sample was used to transfect E. Coli strain BHM71/18 mutL and plaques gridded and grown up as infected colonies.

After transfer to nitrocellulose filters, the colonies were probed at room temperature with 10 pmole of D1.3 CDR1 primer labelled at the 5' end with 30 μCi^{32}-p-ATP. After a 3" wash at 60°C, autoradiography revealed about 20% of the colonies had hybridised well to the probe. All these techniques are fully described in "Oligonucleotide site-directed mutagenesis in M13" an experimental manual by P. Carter, H. Bedouelle, M.M.Y. Waye and G. Winter 1985 and published by Anglian Biotechnology Limited, Hawkins Road, Colchester, Essex CO2 8JX. Several clones were sequenced, and the replacement of HuV_{NP} CDR1 by D.13 CDR1 was confirmed. This M13 template was used in a second round of mutagenesis with D1.3 CDR2 primer; finally template with both CDRs 1&2 replaced was used in a third round of mutagenesis with D.13 CDR3 primer. In this case, three rounds of mutagenesis were used.

The variable domain containing the D1.3 CDRs was then attached to sequences encoding the heavy chain constant regions of human IgG2 so as to produce a vector encoding a heavy chain Hu*. The vector was transfected into J558L cells as above. The antibody $Hu^*_2L_2$ is secreted.

For comparative purposes, the variable region gene for the D1.3 antibody was inserted into a suitable vector and attached to a gene encoding the constant regions of mouse IgG1 to produce a gene encoding a heavy chain H* with the same sequence as H. The protocol for achieving this is shown in Figure 8.

As shown in Figure 8, the gene encoding the D1.3 heavy chain V and C_H1 domains and part of the hinge region are cloned into the M13mp9 vector.

The vector (vector A) is then cut with NcoI, blunted with Klenow polymerase and cut with PstI. The PStI-NcoI fragment is purified and cloned into PstI-HindII cut MV_{NP} vector to replace most of the MV_{NP} coding sequences. The MV_{NP} vector comprises the mouse variable domain gene with its promoter, 5' leader, and 5' and 3' introns cloned into M13mp9. This product is shown as vector B in Figure 8.

Using site directed mutagenesis on the single stranded template of vector B with two primers, the sequence encoding the N-terminal portion of the C_H1 domain and the PstI site near the N-terminus of the V domain are removed. Thus the V domain of D1.3 now replaces that of V_{NP} to produce vector C of Figure 8.

Vector C is then cut with HindIII and BamHI and the fragment formed thereby is inserted into HindIII/BamHI cut M13mp9. The product is cut with Hind III and SacI and the fragment is inserted into PSV-V_{NP} cut with Hind III/SacI so as to replace the V_{NP} variable domain with the D1.3 variable domain. Mouse IgG1 constant domains are cloned into the vector as a SacI fragment to produce vector D of Figure 8.

Vector D of Figure 8 is transfected into J558L cells and the heavy chain H* is secreted in association with the lambda light chain L as an antibody $H^*{}_2L_2$.

Separated K or L light chains can be produced by treating an appropriate antibody (for instance D1.3 antibody to produce K light chains) with 2-mercaptoethanol in guanidine hydrochloride, blocking the free interchain sulphydryls with iodoacetamide and separating the dissociated heavy and light chains by HPLC in guanidine hydrochloride.

Different heavy and light chains can be reassociated to produce functional antibodies by mixing the separated heavy and light chains, and dialysing into a non-denaturing buffer to promote re-association and refolding. Properly reassociated and folded antibody molecules can be purified on protein A-sepharose columns. Using appropriate combinations of the above procedures, the following antibodies were prepared.

H_2K_2	(Dl 3 antibody)
$H^*{}_2L_2$	(Dl.3 heavy chain, lambda light chain)
$H^*{}_2K_2$	(recombinant equivalent of Dl.3)
$Hu^*{}_2L_2$	("humanised" Dl.3 heavy chain, lambda light chain)
$Hu^*{}_2K_2$	("humanised" Dl.3)

The antibodies containing the lambda light chains were not tested for antigen binding capacity. The other antibodies were, and the results are shown in Table 2.

Table 2

Antibody	Dissociation constant for lysozyme (nM)
Dl.3 (H_2K_2)	14.4
Dl.3 (H_2K_2) (reassociated)	15.9, 11.4

recombinant D1.3 ($H^*{}_2K_2$) (reassociated)	9.2
"humanised" D1.3 (Hu_2K_2) (reassociated)	3.5, 3.7

The affinity of the antibodies for lysozyme was determined by fluorescent quenching, with excitation at 290nm and emission observed at 340nm Antibody solutions were diluted to 15-30μg/mg in phosphate buffered saline, filtered (0.45 um-cellulose acetate) and titrated with hen eggwhite lysozyme. There is a quenching of fluorescence on adding the lysozyme to the antibody (>100% quench) and data were fitted by least squares to an equation describing tight binding inhibition (I.H. Segal in Enzyme Kinetics, p73-74, Wiley, New York 1975). Although at first sight the data suggest that the binding of the "humanised" antibody to lysozyme is tighter than in the original D1.3 antibody, this remains to be confirmed. It is clear however that the humanised antibody binds lysozyme with a comparable affinity to D1.3

Further work (with another antibody-CAMPATH1) has shown that CDRs 1,2 and 3 can be exchanged simultaneously, by priming as above with all three primers. 10% hybridisation positives were detected by screening with the CDR1 primer; 30% of these comprised the triple mutant in which all the CDRs were replaced.

It has therefore been shown that CDR replacement can be used not only for artificial antigens (haptens) but also for natural antigens, thereby showing that the present invention will be of therapeutic use.

It will of course be understood that the present invention has been described above purely by way of example, and modifications of detail can be made within the scope of the invention as defined in the appended claims.

CLAIMS

1. An altered antibody in which at least parts of the complementarity determining regions (CDRs) in the light or heavy chain variable domains have been replaced by analogous parts of CDRs from an antibody of different specificity.

2. The altered antibody of claim 1, in which the entire CDRs have been replaced.

3. The altered antibody of claim 1 or claim 2, in which the variable domains in both the heavy and light chains have been altered by CDR replacement.

4. The altered antibody of any one of claims 1 to 3 in which the CDRs from a mouse antibody are grafted onto the framework regions of a human antibody.

5. The altered antibody of any one of claims 1 to 4, which has the structure of a natural antibody or a fragment thereof.

6. A method for producing an altered antibody comprising:

a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least a variable domain of an Ig heavy or light chain, the variable domain comprising framework regions from a first antibody and CDRs comprising at least parts of the CDRs from a second antibody of different specificity;

b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary Ig light or heavy chain respectively;

c) transforming a cell line with the first or both prepared vectors; and

d) culturing said transformed cell line to produce said altered antibody.

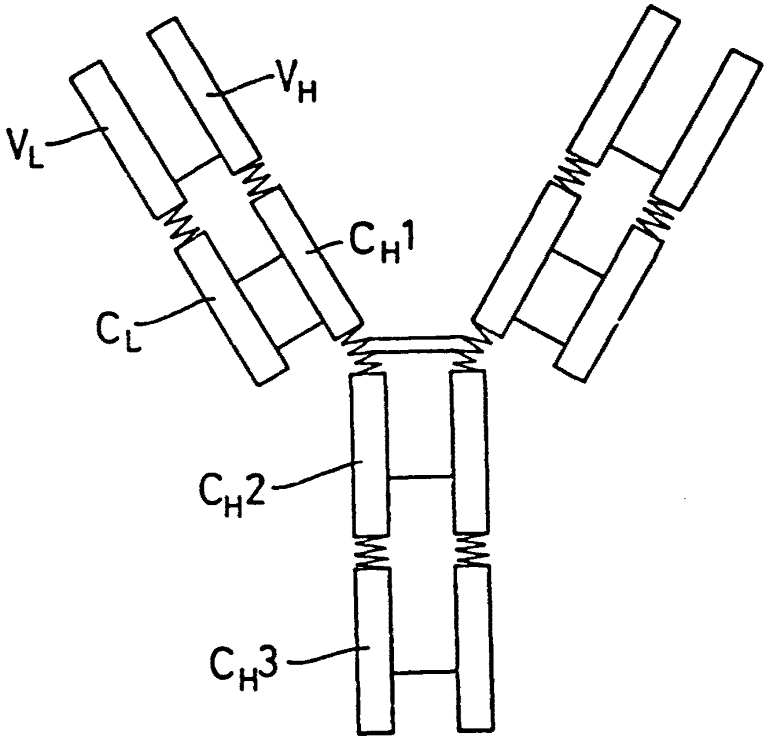
7. The method of claim 6, in which the cell line which is transformed to produce the altered antibody is an immortalised mammalian cell line.

8. The method of claim 7, in which the immortalised cell line is a myeloma cell line or a derivative thereof.

9. The method of any one of claims 6 to 8, in which the DNA sequence encoding the altered variable domain is prepared by oligonucleotide synthesis.

10. The method of any one of claims 6 to 8, in which the DNA sequence encoding the altered variable domain is prepared by primer directed oligonucleotide site-directed mutagenesis using a long oligonucleotide.

Fig. 1



▭ = domains
⋀⋀ = inter-domain sections
— = disulphide bonds
V = variable
C = constant
L = light chain
H = heavy chain

	1 FR1 30	31 CDR1 35
NEWM	XVQLQESGPGLVRPSQTLSLTCTVSGSTFS	NDYYT
B1-8	QVQLQQPGAELVKPGASVKLSCKASGYTFT	SYWMH

	36 FR2 49	50 CDR2 65
NEWM	WVRQPPGRGLEWIG	YVFYHGTSDDTTPLRS
B1-8	WVKQRPGRGLEWIG	RIDPNSGGTKYNEKFKS

	66 FR3 94	95 CDR3 102
NEWM	RVTMLVDTSKNQFSLRLSSVTAADTAVYYCAR	NLIAGCIDV
B1-8	KATLTVDKPSSTAYMQLSSLTSEDSAVYYCAR	YDYYGSSYFDY

	103 FR4 113
NEWM	WGQGSLVTVSS
B1-8	WGQGTTLTVSS

Fig. 2

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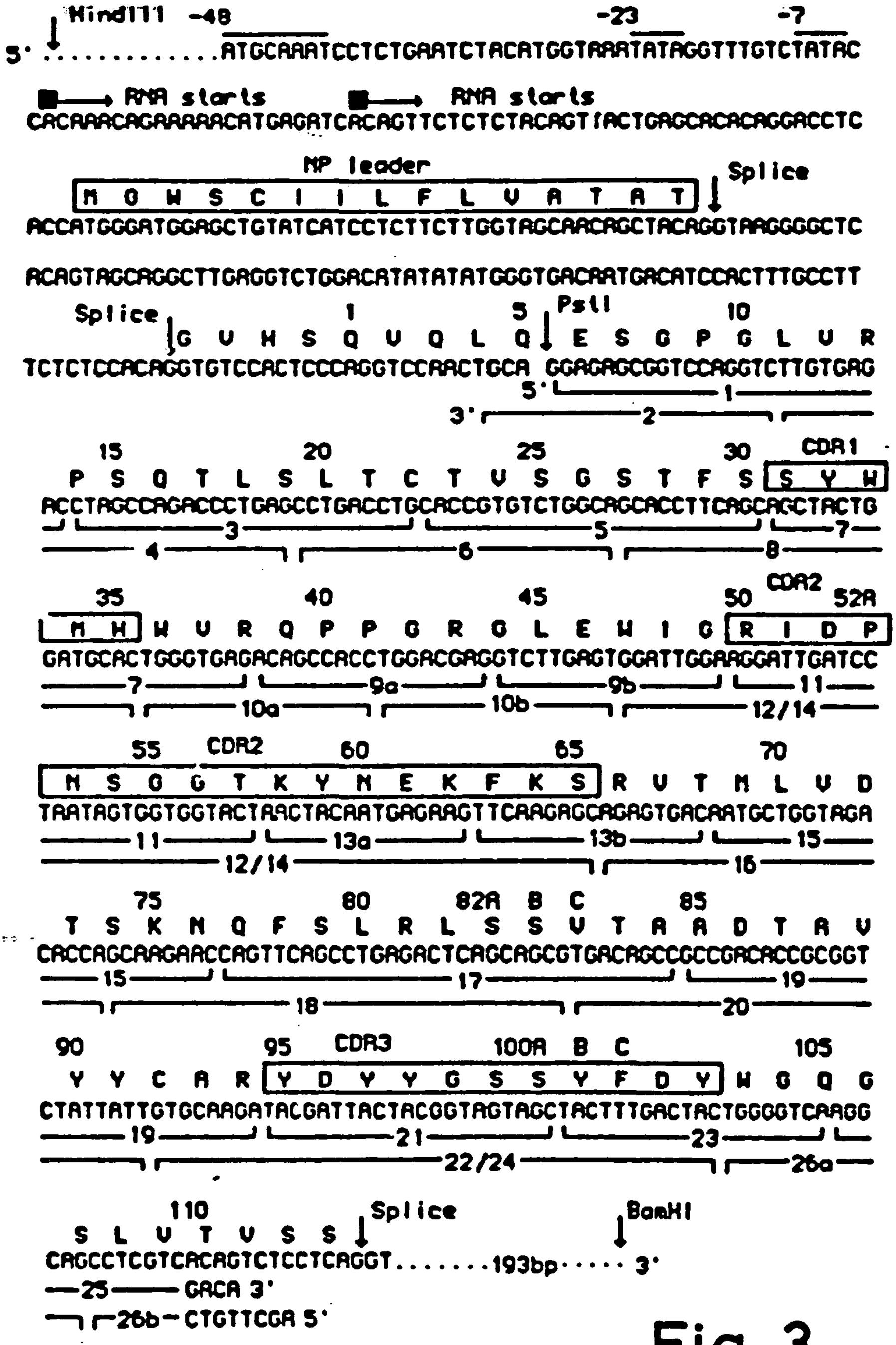
HindIII
-48
-23
-7
5' .............ATGCAAATCCTCTGAATCTACATGGTAAATATAGGTTTGTCTATAC
RNA starts
RNA starts
CACAAACAGAAAAACATGAGATCACAGTTCTCTCTACAGTTACTGAGCACACAGGACCTC
NP leader
M G W S C I I L F L V A T A T
Splice
ACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTAAGGGGCTC
ACAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTT
Splice
1
5
PstI
10
G V H S Q V Q L Q E S G P G L V R
TCTCCACAGGTGTCCACTCCCAGGTCCAACTGCA GGAGAGCGGTCCAGGTCTTGTGAG
5'
1
3'
2
15
20
25
30
CDR1
P S Q T L S L T C T V S G S T F S S Y W
ACCTAGCCAGACCCTGAGCCTGACCTGCACCGTGTCTGGCAGCACCTTCAGCAGCTACTG
3
5
7
4
6
8
35
40
45
50
CDR2
52A
M H W V R Q P P G R G L E W I G R I D P
GATGCACTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGAAGGATTGATCC
7
9a
9b
11
10a
10b
12/14
55
CDR2
60
65
70
N S G G T K Y N E K F K S R V T M L V D
TAATAGTGGTGGTACTAACTACAATGAGAAGTTCAAGAGCAGAGTGACAATGCTGGTAGA
11
13a
13b
15
12/14
16
75
80
82A B C
85
T S K N Q F S L R L S S V T A A D T A V
CACCAGCAAGAACCAGTTCAGCCTGAGACTCAGCAGCGTGACAGCCGCCGACACCGCGGT
15
17
19
18
20
90
95
CDR3
100A B C
105
Y Y C A R Y D Y Y G S S Y F D Y W G Q G
CTATTATTGTGCAAGATACGATTACTACGGTAGTAGCTACTTTGACTACTGGGGTCAAGG
19
21
23
22/24
26a
110
S L V T V S S
Splice
BamHI
CAGCCTCGTCACAGTCTCCTCAGGT.......193bp····· 3'
25 GACA 3'
26b CTGTTCGA 5'

Fig. 3

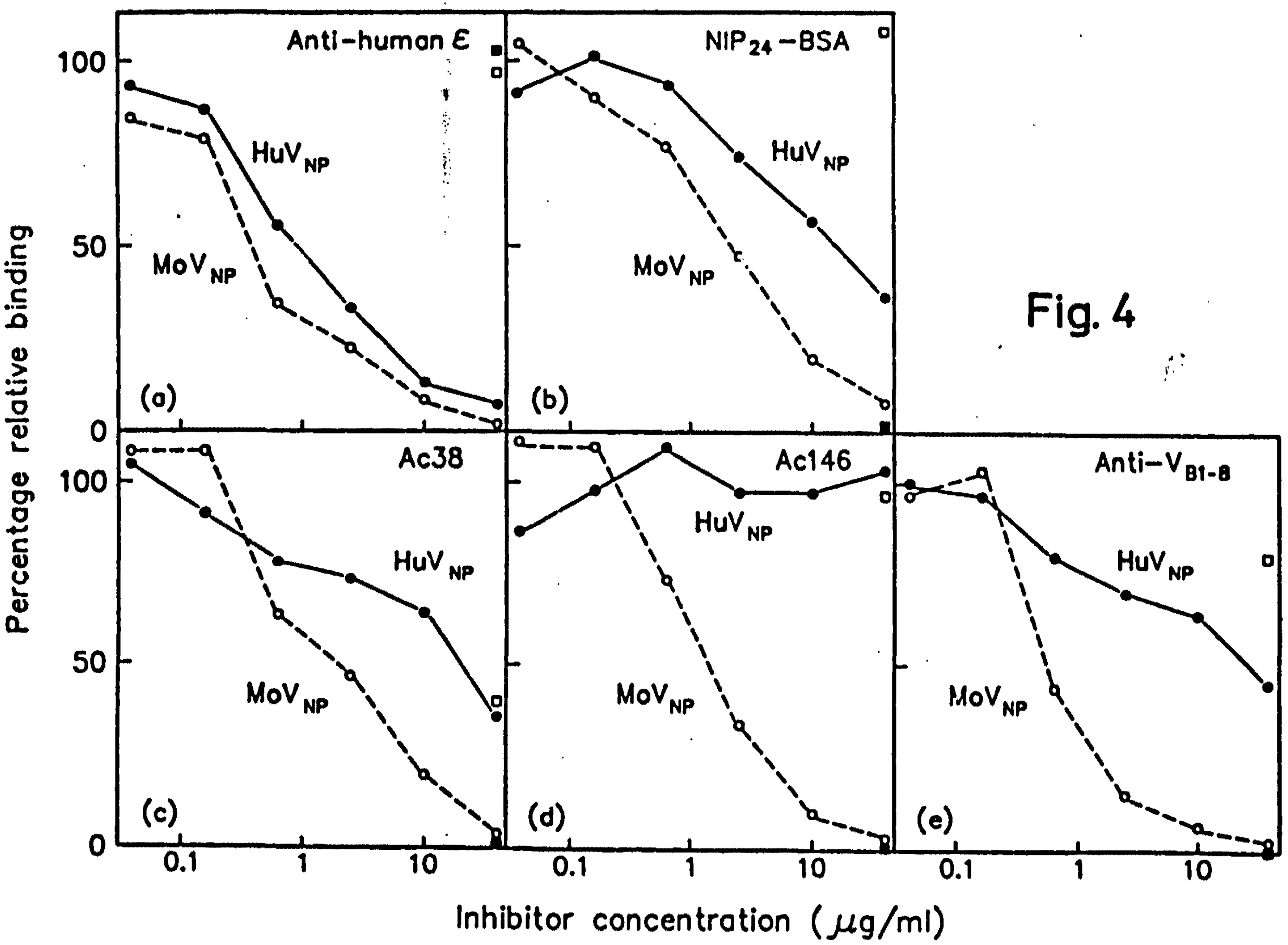

Fig. 4

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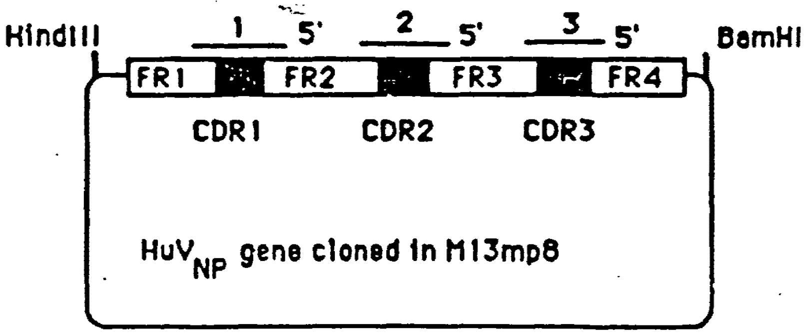
Fig. 5



D1.3 CDR1 oligonucleotide

5' CTG,TCT,CAC,CCA,GTT,TAC,ACC,ATA,GCC,GCT,GAA,GGT,GCT

FR2 D1.3 CDR1 FR1

D1.3 CDR2 oligonucleotide

5' CAT,TGT,CAC,TCT,GGA,TTT,GAG,AGC,TGA,ATT,ATA,GTC,TGT,

FR3 D1.3 CDR2

GTT,TCC,ATC,ACC,CCA,AAT,CAT,TCC,AAT,CCA,CTC

D1.3 CDR2 FR2

D1.3 CDR3 oligonucleotide

5' GCC,TTG,ACC,CCA,GTA,GTC,AAG,CCT,ATA,ATC,TCT,CTC,TCT,

FR4 D1.3 CDR3

TGC,ACA,ATA

FR3

Fig. 6.

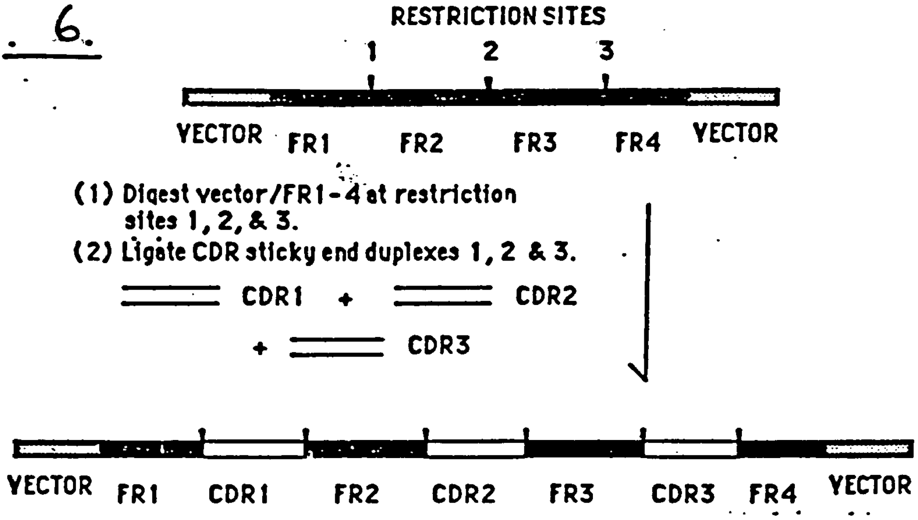
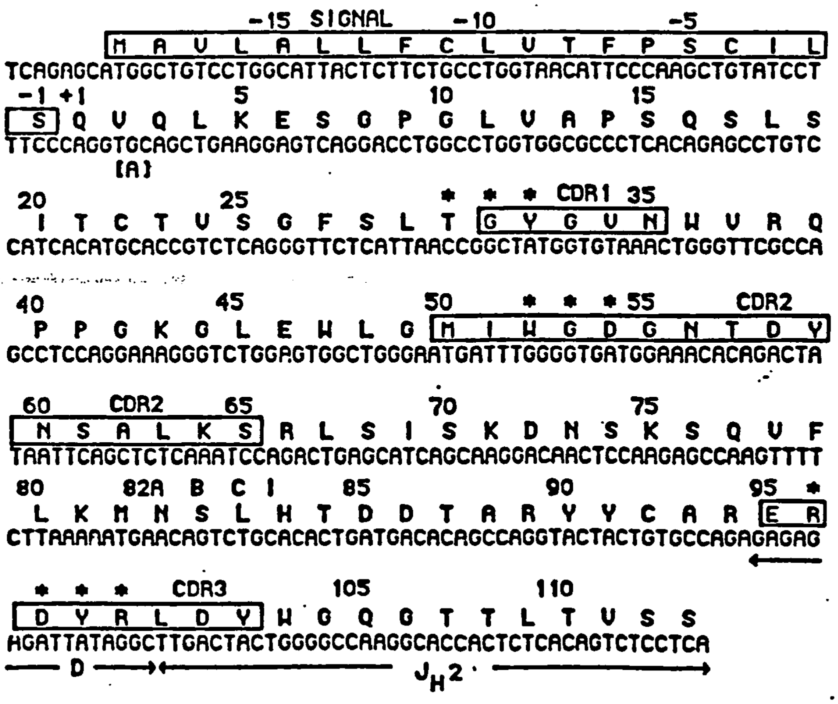


Fig. 7.

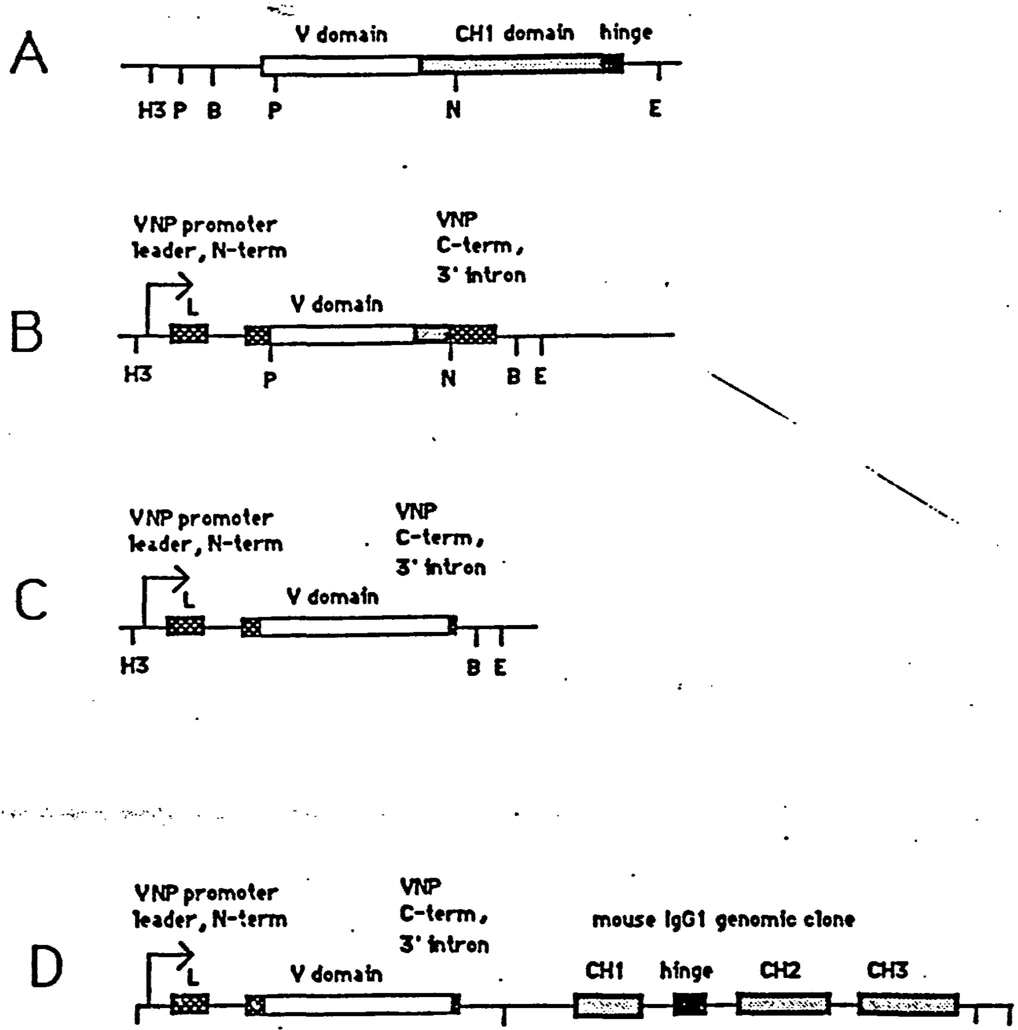


```
                 -15 SIGNAL    -10          -5
         [M  A  V  L  A  L  L  F  C  L  U  T  F  P  S  C  I  L]
TCAGAGCATGGCTGTCCTGGCATTACTCTTCTGCCTGGTAACATTCCCAAGCTGTATCCT
 -1 +1       5              10             15
[S] Q  U  Q  L  K  E  S  G  P  G  L  U  A  P  S  Q  S  L  S
TTCCCAGGTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGCCTGTC
     [A]
 20             25             *  *  * CDR1 35
 I  T  C  T  U  S  G  F  S  L  T [G  Y  G  U  N] H  U  R  Q
CATCACATGCACCGTCTCAGGGTTCTCATTAACCGGCTATGGTGTAAACTGGGTTCGCCA

 40             45             50    *  *  * 55       CDR2
 P  P  G  K  G  L  E  H  L  G [M  I  H  G  D  G  N  T  D  Y]
GCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAATGATTTGGGGTGATGGAAACACAGACTA

 60    CDR2     65             70             75
[N  S  A  L  K  S] R  L  S  I  S  K  D  N  S  K  S  Q  U  F
TAATTCAGCTCTCAAATCCAGACTGAGCATCAGCAAGGACAACTCCAAGAGCCAAGTTTT
 80      82A B  C  I     85                90             95 *
 L  K  M  N  S  L  H  T  D  D  T  A  R  Y  Y  C  A  R [E  R]
CTTAAAAATGAACAGTCTGCACACTGATGACACAGCCAGGTACTACTGTGCCAGAGAGAG
                                                        <----
 *  *  *     CDR3          105             110
[D  Y  R  L  D  Y] H  G  Q  G  T  T  L  T  U  S  S
AGATTATAGGCTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
---- D --->|<--------------- JH2 ---------------->
```

Fig. 8.



H3 = HindIII, P = PstI, B = BamHI, N = NcoI, E = EcoRI, H2 = HindII

⑲ Europäisches Patentamt
European Patent Office
Office européen des brevets

⑪ Publication number: **0 620 276 A1**

⑫ **EUROPEAN PATENT APPLICATION**

㉑ Application number: 94104042.0

㉒ Date of filing: 21.12.90

(51) Int. Cl.5: C12N 15/13, C07K 15/28,
A61K 39/395, G01N 33/577

This application was filed on 16 - 03 - 1994 as a divisional application to the application mentioned under INID code 60.

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

㉚ Priority: 21.12.89 GB 8928874

㊸ Date of publication of application:
19.10.94 Bulletin 94/42

(60) Publication number of the earlier application in accordance with Art.76 EPC: 0 460 167

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

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(54) Humanised antibodies.

(57) CDR-grafted antibody heavy and light chains comprise acceptor framework and donor antigen binding regions, the heavy chains comprising donor residues at at least one of positions (6, 23) and/or (24, 48) and/or (49, 71) and/or (73, 75) and/or (76) and/or (78) and/or (91). The CDR-grated light chains comprise donor residues at at least one of positions (1) and/or (3) and (46) and/or (47) or at at least one of positions (46, 48, 58) and (71). The CDR-grafted antibodies are preferably humanised antibodies, having non-human, e.g. rodent, donor residues and human acceptor frameworks, and may be used for *in vivo* therapy and diagnosis. A generally applicable protocol is disclosed for obtaining CDR-grafted antibodies.

Rank Xerox (UK) Business Services
(3.10/3.09/3.3.4)

Carter et al. P0709P1
SN: 08/146,206
Filed November 17, 1993

```
  1 GAATTCCCAA AGACAAAatg gattttcaag tgcagatttt cagcttcctg
 51 ctaatcagtg cctcagtcat aatatccaga ggacaaattg ttctcaccca
101 gtctccagca atcatgtctg catctccagg ggagaaggtc accatgacct
151 gcagtgccag ctcaagtgta agttacatga actggtacca gcagaagtca
201 ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
251 agtccctgct cacttcaggg gcagtgggtc tgggacctct tactctctca
301 caatcagcgg catggaggct gaagatgctg ccacttatta ctgccagcag
351 tggagtagta acccattcac gttcggctcg gggacaaagt tggaaataaa
401 ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
451 agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
501 cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
551 tggcgtcctg aacagttgga ctgatcagga cagcaaagac agcacctaca
601 gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
651 agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
701 gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
751 CCAGCTCCCA GCTCCATCCT ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
801 CCACAAGCGC tTACCACTGT TGCGGTGCTC tAAACCTCCT CCCACCTCCT
851 TCTCCTCCTC CTCCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
901 AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA
```

Fig. 1(a)

```
  1 MDFQVQIFSF LLISASVIIS RGQIVLTQSP AIMSASPGEK VTMTCSASSS
 51 VSYMNWYQQK SGTSPKRWIY DTSKLASGVP AHFRGSGSGT SYSLTISGME
101 AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
151 ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
201 TLTKDEYERH NSYTCEATHK TSTSPIVKSF NRNEC*
```

Fig. 1(b)

Field of the Invention

The present invention relates to humanised antibody molecules, to processes for their production using recombinant DNA technology, and to their therapeutic uses.

The term "humanised antibody molecule" in used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site typically comprises complementarity determining regions (CDRs) which determine the binding specificity of the antibody molecule and which are carried on appropriate framework regions in the variable domains. There are 3 CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Background of the Invention

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, $(Fab')_2$ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, were hindered until recently by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies (MAbs) of defined specificity (1).

However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness. In practice, MAbs of rodent origin may not be used in patients for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. For instance, OKT3 a mouse IgG2a/k MAb which recognises an antigen in the T-cell receptor-CD3 complex has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection [Chatenoud et al (2) and Jeffers et al (3)]. However, in view of the rodent nature of this and other such MAbs, a significant HAMA response which may include a major anti-idiotype component, may build up on use. Clearly, it would be highly desirable to diminish or abolish this undesirable HAMA response and thus enlarge the areas of use of these very useful antibodies.

Proposals have therefore been made to render non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These techniques typically involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MAbs involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP0120694 (Celltech Limited), EP0125023 (Genentech Inc. and City of Hope), EP-A-0 171496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), and WO 86/01533 (Celltech Limited). This latter Celltech application (WO 86/01533) discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. Such humanized chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period [Begent et al (ref. 4)].

In an alternative approach, described in EP-A-0239400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The present invention relates to humanized antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanized antibodies are much less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the much lower proportion of non-human amino acid sequence which they contain.

The earliest work on humanizing MAbs by CDR-grafting was carried out on MAbs recognizing synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAb recognizing lysozyme and a rat MAb recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen et al (5) and Riechmann et al (6) respectively. The preparation of CDR-grafted antibody to the antigen on human T cells is also described in WO 89/07452 (Medical Research Council).

In Riechmann et al/Medical Research Council it was found that transfer of the CDR regions alone [as defined by Kabat refs. (7) and (8)] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having improved antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanised antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, in particular in the structural loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognise more complex antigens. Even so the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAb.

Very recently Queen et al (9) have described the preparation of a humanised antibody that binds to the interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. The human framework regions were chosen to maximise homology with the anti-Tac MAb sequence. In addition computer modelling was used to identify framework amino acid residues which wore likely to interact with the CDRs or antigen, and mouse amino acids were used at these positions in the humanised antibody.

In WO 90/07861 Queen et al propose four criteria for designing humanised immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is unusually homologous to the non-human donor immunoglobulin to be humanised, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanised immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, and may be applied singly or in any combination.

WO 90/07861 describes in detail the preparation of a single CDR-grafted humanised antibody, a humanised antibody having specificity for the p55 Tac protein of the IL-2 receptor. The combination of all four criteria, as above, were employed in designing this humanized antibody, the variable region frameworks of the human antibody Eu (7) being used as acceptor. In the resultant humanised antibody the donor CDRs were as defined by Kabat et al (7 and 8) and in addition the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in the heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanised anti-Tac antibody obtained is reported to have an affinity for p55 of 3 x 10^9 M^{-1}, about one-third of that of the murine MAb.

We have further investigated the preparation of CDR-grafted humanised antibody molecules and have identified a hierarchy of positions within the framework of the variable regions (i.e. outside both the Kabat CDRs and structural loops of the variable regions) at which the amino acid identities of the residues are important for obtaining CDR-grafted products with satisfactory binding affinity. This has enabled us to establish a protocol for obtaining satisfactory CDR-grafted products which may be applied very widely irrespective of the level of homology between the donor immunoglobulin and acceptor framework. The set of residues which we have identified as being of critical importance does not coincide with the residues identified by Queen et al (9).

Summary of the Invention

Accordingly, in a first aspect the invention provides a CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

In preferred embodiments, the heavy chain framework comprises donor residues at positions 23, 24, 49, 71, 73 and 78 or at positions 23, 24 and 49. The residues at positions 71, 73 and 78 of the heavy chain framework are preferably either all acceptor or all donor residues.

In particularly preferred embodiments the heavy chain framework additionally comprises donor residues at one, some or all of positions 6, 37, 48 and 94. Also it is particularly preferred that residues at positions of the heavy chain framework which are commonly conserved across species, i.e. positions 2, 4, 25, 36, 39, 47, 93, 103, 104, 106 and 107, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the heavy chain framework additionally comprises donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

In addition the heavy chain framework optionally comprises donor residues at one, some or all of positions:
1 and 3,
72 and 76,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
80 and 20 (if 69 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91,
88, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

In the first and other aspects of the present invention reference is made to CDR-grafted antibody products comprising acceptor framework and donor antigen binding regions. It will be appreciated that the invention is widely applicable to the CDR-grafting of antibodies in general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

In the first and other aspects of the present invention, the donor antigen binding region typically comprises at least one CDR from the donor antibody. Usually the donor antigen binding region comprises at least two and preferably all three CDRs of each of the heavy chain and/or light chain variable regions. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a composite of the Kabat and structural loop CDRs and any combination of any of these. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR2 (residues 50-65) and CDR3 (residues 95-100) and a composite of the Kabat and structural loop CDRs at CDR1 (residues 26-35).

The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering [refs. (7) and (8)]. Thus the residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. For example, the heavy chain variable region of the anti-Tac antibody described by Queen et al (9) contains a single amino acid insert (residue 52a) after residue 52 of CDR2 and a three amino acid insert (residues 82a, 82b and 82c) after framework residue 82, in the Kabat numbering. The correct Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The invention also provides in a second aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47. Preferably the CDR grafted light chain of the second aspect comprises donor residues at positions 46 and/or 47.

The invention also provides in a third aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

In a preferred embodiment of the third aspect, the framework comprises donor residues at all of positions 46, 48, 58 and 71.

In particularly preferred embodiments of the second and third aspects, the framework additionally comprises donor residues at positions 36, 44, 47, 85 and 87. Similarly positions of the light chain framework which are commonly conserved across species, i.e. positions 2, 4, 6, 35, 49, 62, 64-69, 98, 99, 101 and 102, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the light chain framework additionally comprises donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

In addition the framework of the second or third aspects optionally comprises donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form at potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 is different between donor and acceptor),
and
any one or more of 10, 12, 40, 80, 103 and 105.

Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR1 (residue 24-34), CDR2 (residues 50-56) and CDR3 (residues 89-97).

The invention further provides in a fourth aspect a CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain and at least one CDR-grafted light chain according to the first and second or first and third aspects of the invention.

The humanised antibody molecules and chains of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, (Fab')2 or FV fragment; a light chain or heavy chain monomer or dimer; or a single chain antibody, e.g. a single chain FV in which heavy and light chain variable regions are joined by a peptide linker; or any other CDR-grafted molecule with the same specificity as the original donor antibody. Similarly the CDR-grafted heavy and light chain variable region may be combined with other antibody domains as appropriate.

Also the heavy or light chains or humanised antibody molecules of the present invention may have attached to them an effector or reporter molecule. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce an immunoglobulin molecule in which the Fc fragment or CH3 domain of a complete immunoglobulin molecule has been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

Any appropriate acceptor variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived. Preferably, the type of acceptor framework used is of the same/similar class/type as the donor antibody. Conveniently, the framework may be chosen to maximise/optimise homology with the donor antibody sequence particularly at positions close or adjacent to the CDRs. However, a high level of homology between donor and acceptor sequences is not important for application of the present invention. The present invention identifies a hierarchy of framework residue positions at which donor residues may be important or desirable for obtaining a CDR-grafted antibody product having satisfactory binding properties. The CDR-grafted products usually have binding affinities of at least 10^5 M^{-1}, preferably at least about 10^8 M^{-1}, or especially in the range 10^8-10^{12} M^{-1}. In principle, the present invention is applicable to any combination of donor and acceptor antibodies irrespective of the level of homology between their sequences. A protocol for applying the invention to any particular donor-acceptor antibody pair is given hereinafter. Examples of human frameworks which may be used are KOL, NEWM, REI, EU, LAY and POM (refs. 4 and 5) and the like; for instance KOL and NEWM for the heavy chain and REI for the light chain and EU, LAY and POM for both the heavy chain and the light chain.

Also the constant region domains of the products of the invention may be selected having regard to the proposed function of the antibody in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgE, IgG or IgM domains. In particular, IgG human constant region domains may be used, especially of the IgG1 and IgG3 isotypes, when the humanised antibody molecule is intended for therapeutic uses, and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the humanised antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simple blocking of lymphokine activity.

However, the remainder of the antibody molecules need not comprise only protein sequences from immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a functional polypeptide such as an effector or reporter molecule.

Preferably the CDR-grafted antibody heavy and light chain and antibody molecule products are produced by recombinant DNA technology.

Thus in further aspects the invention also includes DNA sequences coding for the CDR-grafted heavy and light chains, cloning and expression vectors containing the DNA sequences, host cells transformed with the DNA sequences and processes for producing the CDR-grafted chains and antibody molecules comprising expressing the DNA sequences in the transformed host cells.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 10 and 11.

The DNA sequences which encode the donor amino acid sequence may be obtained by methods well known in the art. For example the donor coding sequences may be obtained by genomic cloning, or cDNA cloning from suitable hybridoma cell lines. Positive clones may be screened using appropriate probes for the heavy and light chain genes in question. Also PCR cloning may be used.

DNA coding for acceptor, e.g. human acceptor, sequences may be obtained in any appropriate way. For example DNA sequences coding for preferred human acceptor frameworks such as KOL, REI, EU and NEWM, are widely available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example oligonucleotide directed synthesis as described by Jones et al (ref. 20) may be used. Also oligonucleotide directed mutagenesis of a pre-exising variable region as, for example, described by Verhoeyen et al (ref. 5) or Riechmann et al (ref. 6) may be used. Also enzymatic filling in of gapped oligonucleotides using T_4 DNA polymerase as, for example, described by Queen et al (ref. 9) may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial e.g. E. coli, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and (Fab')$_2$ fragments, and especially FV fragments and single chain antibody fragments e.g. single chain FVs. Eucaryotic e.g. mammalian host cell expression systems may be used for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Thus, in a further aspect the present invention provides a process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to the first aspect of the invention;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to the second or third aspect of the invention;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

The CDR-grafted product may comprise only heavy or light chain derived polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence is used to transfect the host cells. For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, the first vector may contain an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical, except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially, genomic DNA, preferably a fusion of cDNA and genomic DNA.

The present invention is applicable to antibodies of any appropriate specificity. Advantageously, however, the invention may be applied to the humanisation of non-human antibodies which are used for in vivo therapy or diagnosis. Thus the antibodies may be site-specific antibodies such as tumour-specific or cell surface-specific antibodies, suitable for use in in vivo therapy or diagnosis, e.g. tumour imaging. Examples of cell surface-specific antibodies are anti-T cell antibodies, such as anti-CD3, and CD4 and adhesion molecules, such as CR3, ICAM and ELAM. The antibodies may have specificity for interleukins (including lymphokines, growth factors and stimulating factors), hormones and other biologically active compounds, and receptors for any of these. For example, the antibodies may have specificity for any of the following: Interferons α, β, γ or δ, IL1, IL2, IL3, or IL4, etc., TNF, GCSF, GMCSF, EPO, hGH, or insulin, etc.

The the present invention also includes therapeutic and diagnostic compositions comprising the CDR-grafted products of the invention and uses of such compositions in therapy and diagnosis.

Accordingly in a further aspect the invention provides a therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Accordingly also the invention provides a method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention to a human or animal subject.

A preferred protocol for obtaining CDR-grafted antibody heavy and light chains in accordance with the present invention is set out below together with the rationale by which we have derived this protocol. This protocol and rationale are given without prejudice to the generality of the invention as hereinbefore described and defined.

Protocol

It is first of all necessary to sequence the DNA coding for the heavy and light chain variable regions of the donor antibody, to determine their amino acid sequences. It is also necessary to choose appropriate acceptor heavy and light chain variable regions, of known amino acid sequence. The CDR-grafted chain is then designed starting from the basis of the acceptor sequence. It will be appreciated that in some cases the donor and acceptor amino acid residues may be identical at a particular position and thus no change of acceptor framework residue is required.

1. As a first step donor residues are substituted for acceptor residues in the CDRs. For this purpose the CDRs are preferably defined as follows:

Heavy chain	- CDR1: residues 26-35
	- CDR2: residues 50-65
	- CDR3: residues 95-102
Light chain	- CDR1: residues 24-34
	- CDR2: residues 50-56
	- CDR3: residues 89-97

The positions at which donor residues are to be substituted for acceptor in the framework are then chosen as follows, first of all with respect to the heavy chain and subsequently with respect to the light chain.

2. Heavy Chain

2.1 Choose donor residues at all of positions 23, 24, 49, 71, 73 and 78 of the heavy chain or all of positions 23, 24 and 49 (71, 73 and 78 are always either all donor or all acceptor).

2.2 Check that the following have the same amino acid in donor and acceptor sequences, and if not preferably choose the donor: 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

2.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 72, 76
iii. If 48 is different between donor and acceptor sequences, consider 69
iv. If at 48 the donor residue is chosen, consider 38 and 46
v. If at 69 the donor residue is chosen, consider 80 and then 20
vi. 67
vii. If at 67 the donor residue is chosen, consider 82 and then 18
viii. 91
ix. 88
x. 9, 11, 41, 87, 108, 110, 112

3. Light Chain

3.1 Choose donor at 46, 48, 58 and 71

3.2 Check that the following have the same amino acid in donor and acceptor sequences, if not preferably choose donor:

2, 4, 6, 35, 38, 44, 47, 49, 62, 64-69 inclusive, 85, 87, 98, 99, 101 and 102

3.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 63
iii. 60, if 60 and 54 are able to form potential saltbridge
iv. 70, if 70 and 24 are able to form potential saltbridge
v. 73, and 21 if 47 is different between donor and acceptor
vi. 37, and 45 if 47 is different between donor and acceptor
vii. 10, 12, 40, 80, 103, 105

Rationale

In order to transfer the binding site of an antibody into a different acceptor framework, a number of factors need to be considered.

1. The extent of the CDRs

The CDRs (Complementary Determining Regions) were defined by Wu and Kabat (refs. 4 and 5) on the basis of an analysis of the variability of different regions of antibody variable regions. Three regions per domain were recognised. In the light chain the sequences are 24-34, 50-56, 89-97 (numbering according to Kabat (ref. 4), Eu Index) inclusive and in the heavy chain the sequences are 31-35, 50-65 and 95-102 inclusive.

When antibody structures became available it became apparent that these CDR regions corresponded in the main to loop regions which extended from the β barrel framework of the light and heavy variable domains. For H1 there was a discrepancy in that the loop was from 26 to 32 inclusive and for H2 the loop was 52 to 56 and for L2 from 50 to 53. However, with the exception of H1 the CDR regions encompassed the loop regions and extended into the β strand frameworks. In H1 residue 26 tends to be a serine and 27 a phenylalanine or tyrosine, residue 29 is a phenylalanine in most cases. Residues 28 and 30 which are surface residues exposed to solvent might be involved in antigen-binding. A prudent definition of the H1 CDR therefore would include residues 26-35 to include both the loop region and the hypervariable residues 33-35.

It is of interest to note the example of Riechmann et al (ref. 3), who used the residue 31-35 choice for CDR-H1. In order to produce efficient antigen binding, residue 27 also needed to be recruited from the donor (rat) antibody.

2. Non-CDR residues which contribute to antigen binding

By examination of available X-ray structures we have identified a number of residues which may have an effect on net antigen binding and which can be demonstrated by experiment. These residues can be sub-divided into a number of groups.

2.1 Surface residues near CDR [all numbering as in Kabat et al (ref. 7)].

2.1.1. Heavy Chain - Key residues are 23, 71 and 73. Other residues which may contribute to a lesser extent are 1, 3 and 76. Finally 25 is usually conserved but the murine residue should be used if there is a difference.

2.1.2 Light Chain - Many residues close to the CDRs, e.g. 63, 65, 67 and 69 are conserved. If conserved none of the surface residues in the light chain are likely to have a major effect. However, if the murine residue at these positions is unusual, then it would be of benefit to analyse the likely contribution more closely. Other residues which may also contribute to binding are 1 and 3, and also 60 and 70 if the residues at these positions and at 54 and 24 respectively are potentially able to form a salt bridge i.e. 60 + 54; 70 + 24.

2.2 Packing residues near the CDRs.

2.2.1. Heavy Chain - Key residues are 24, 49 and 78. Other key residues would be 36 if not a tryptophan, 94 if not an arginine, 104 and 106 if not glycines and 107 if not a threonine. Residues which may make a further contribution to stable packing of the heavy chain and hence improved affinity are 2, 4, 6, 38, 46, 67 and 69. 67 packs against the CDR residue 63 and this pair could be either both mouse or both human. Finally, residues which contribute to packing in this region but from a longer range are 18, 20, 80, 82 and 86. 82 packs against 67 and in turn 18 packs against 82. 80 packs against 69 and in turn 20 packs against 80. 86 forms an H bond network with 38 and 46. Many of the mouse-human differences appear minor e.g. Leu-Ile, but could have an minor impact on correct packing which could translate into altered positioning of the CDRs.

2.2.2. Light Chain - Key residues are 48, 58 and 71. Other key residues would be 6 if not glutamine, 35 if not tryptophan, 62 if not phenylalanine or tryosine, 64, 66, 68, 99 and 101 if not glycines and 102 if not a threonine. Residues which make a further contribution are 2, 4, 37, 45 and 47. Finally residues 73 and 21 and 19 may make long distance packing contributions of a minor nature.

2.3. Residues at the variable domain interface between heavy and light chains - In both the light and heavy chains most of the non-CDR interface residues are conserved. If a conserved residue is replaced by a residue of different character, e.g. size or charge, it should be considered for retention as the murine residue.

2.3.1. Heavy Chain - Residues which need to be considered are 37 if the residue is not a valine but is of larger side chain volume or has a charge or polarity. Other residues are 39 if not a glutamine, 45 if not a leucine, 47 if not a tryptophan, 91 if not a phenylalanine or tyrosine, 93 if not an alanine and 103 if not a tryptophan. Residue 89 is also at the interface but is not in a position where the side chain could be of great impact.

2.3.2. Light Chain - Residues which need to be considered are 36, if not a tyrosine, 38 if not a glutamine, 44 if not a proline, 46, 49 if not a tyrosine, residue 85, residue 87 if not a tyrosine and 98 if not a phenylalanine.

2.4. Variable-Constant region interface - The elbow angle between variable and constant regions may be affected by alterations in packing of key residues in the variable region against the constant region which may affect the position of V_L and V_H with respect to one another. Therefore it is worth noting the residues likely to be in contact with the constant region. In the heavy chain the surface residues potentially in contact with the variable region are conserved between mouse and human antibodies therefore the variable region contact residues may influence the V-C interaction. In the light chain the amino acids found at a number of the constant region contact points vary, and the V & C regions are not in such close proximity as the heavy chain. Therefore the influences of the light chain V-C interface may be minor.

2.4.1. Heavy Chain - Contact residues are 7, 11, 41, 87, 108, 110, 112.

2.4.2. Light Chain - In the light chain potentially contacting residues are 10, 12, 40, 80, 83, 103 and 105.

The above analysis coupled with our considerable practical experimental experience in the CDR-grafting of a number of different antibodies have lead us to the protocol given above.

The present invention is now described, by way of example only, with reference to the accompanying Figures 1 - 13.

Brief Description of the Figures

Figure 1	shows DNA and amino acid sequences of the OKT3 light chain;
Figure 2	shows DNA and amino acid sequences of the OKT3 heavy chain;
Figure 3	shows the alignment of the OKT3 light variable region amino acid sequence with that of the light variable region of the human antibody REI;
Figure 4	shows the alignment of the OKT3 heavy variable region amino acid sequence with that of the heavy variable region of the human antibody KOL;
Figure 5	shows the heavy variable region amino acid sequences of OKT3, KOL and various corresponding CDR grafts;
Figure 6	shows the light variable region amino acid sequences of OKT3, REI and various corresponding CDR grafts;
Figure 7	shows a graph of binding assay results for various grafted OKT3 antibodies'
Figure 8	shows a graph of blocking assay results for various grafted OKT3 antibodies;
Figure 9	shows a similar graph of blocking assay results;
Figure 10	shows similar graphs for both binding assay and blocking assay results;
Figure 11	shows further similar graphs for both binding assay and blocking assay results;
Figure 12	shows a graph of competition assay results for a minimally grafted OKT3 antibody compared with the OKT3 murine reference standard, and
Figure 13	shows a similar graph of competition assay results comparing a fully grafted OKT3 antibody with the murine reference standard.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

EXAMPLE 1

CDR-GRAFTING OF OKT3

MATERIAL AND METHODS

1. INCOMING CELLS

Hybridoma cells producing antibody OKT3 were provided by Ortho (seedlot 4882.1) and were grown up in antibiotic free Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine and 5% foetal calf serum, and divided to provide both an overgrown supernatant for evaluation and cells for extraction of RNA. The overgrown supernatant was shown to contain 250 ug/mL murine IgG2a/kappa antibody. The supernatant was negative for murine lambda light chain and IgG1, IgG2b, IgG3, IgA and IgM heavy chain. 20mL of supernatant was assayed to confirm that the antibody present was OKT3.

2. MOLECULAR BIOLOGY PROCEDURES

Basic molecular biology procedures were as described in Maniatis et al (ref. 9) with, in some cases, minor modifications. DNA sequencing was performed as described in Sanger et al (ref. 11) and the Amersham International Plc sequencing handbook. Site directed mutagenesis was as described in Kramer et al (ref. 12) and the Anglian Biotechnology Ltd. handbook. COS cell expression and metabolic labelling studies were as described in Whittle et al (ref. 13)

3. RESEARCH ASSAYS

3.1. ASSEMBLY ASSAYS Assembly assays were performed on supernatants from transfected COS cells to determine the amount of intact IgG present.

3.1.1. COS CELLS TRANSFECTED WITH MOUSE OKT3 GENES The assembly assay for intact mouse IgG in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-mouse IgG Fc. The plates were washed in water and samples added for 1 hour at room temperature. The plates were washed and F(ab')2 goat anti-mouse IgG F(ab')2 (HRPO conjugated) was then added. Substrate was added to reveal the reaction. UPC10, a mouse IgG2a myeloma, was used as a standard.

3.1.2. COS AND CHO CELLS TRANSFECTED WITH CHIMERIC OR CDR-GRAFTED OKT3 GENES

The assembly assay for chimeric or CDR-grafted antibody in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-human IgG Fc. The plates were washed and samples added and incubated for 1 hour at room temperature. The plates were washed and monoclonal mouse anti-human kappa chain was added for 1 hour at room temperature.

The plates were washed and F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added. Enzyme substrate was added to reveal the reaction. Chimeric B72.3 (IgG4) (ref. 13) was used as a standard. The use of a monoclonal anti-kappa chain in this assay allows grafted antibodies to be read from the chimeric standard.

3.2. ASSAY FOR ANTIGEN BINDING ACTIVITY

Material from COS cell supernatants was assayed for OKT3 antigen binding activity onto CD3 positive cells in a direct assay. The procedure was as follows:

HUT 78 cells (human T cell line, CD3 positive) were maintained in culture. Monolayers of HUT 78 cells were prepared onto 96 well ELISA plates using poly-L-lysine and glutaraldehyde. Samples were added to the monolayers for 1 hour at room temperature.

The plates were washed gently using PBS. F(ab')2 goat anti-human IgG Fc (HRPO conjugated) or F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added as appropriate for humanised or mouse samples. Substrate was added to reveal the reaction.

The negative control for the cell-based assay was chimeric B72.3. The positive control was mouse Orthomune OKT3 or chimeric OKT3, when available. This cell-based assay was difficult to perform, and an alternative assay was developed for CDR-grafted OKT3 which was more sensitive and easier to carry out.

In this system CDR-grafted OKT3 produced by COS cells was tested for its ability to bind to the CD3-positive HPB-ALL (human peripheral blood acute lymphocytic leukemia) cell line. It was also tested for its ability to block the binding of murine OKT3 to these cells. Binding was measured by the following procedure: HPB-ALL cells were harvested from tissue culture. Cells were incubated at 4°C for 1 hour with various dilutions of test antibody, positive control antibody, or negative control antibody. The cells were washed once and incubated at 4°C for 1 hour with an FITC-labelled goat anti-human IgG (Fc-specific, mouse absorbed). The cells were washed twice and analysed by cytofluorography. Chimeric OKT3 was used as a positive control for direct binding. Cells incubated with mock- transfected COS ~~cell supernatant, followed by the FITC-labelled goat anti-human IgG, provided the negative control.~~ To test the ability of CDR-grafted OKT3 to block murine OKT3 binding, the HPB-ALL cells were incubated at 4°C for 1 hour with various dilutions of test antibody or control antibody. A fixed saturating amount of FITC OKT3 was added. The samples were incubated for 1 hour at 4°C, washed twice and analysed by cytofluorography. FITC-labelled OKT3 was used as a positive control to determine maximum binding. Unlabelled murine OKT3 served as a reference standard for blocking. Negative controls were unstained cells with or without mock-transfected cell supernatant. The ability of the CDR-grafted OKT3 light chain to bind CD3-positive cells and block the binding of murine OKT3 was initially tested in combination with the chimeric OKT3 heavy chain. The chimeric OKT3 heavy chain is composed of the murine OKT3 variable region and the human IgG4 constant region. The chimeric heavy chain gene is expressed in the same expression vector used for the CDR-grafted genes. The CDR-grafted light chain expression vector and the chimeric heavy chain expression vector were co-transfected into COS cells. The fully chimeric OKT3 antibody (chimeric light chain and chimeric heavy chain) was found to be fully capable of binding to CD3 positive cells and blocking the binding of murine OKT3 to these cells.

3.3 DETERMINATION OF RELATIVE BINDING AFFINITY

The relative binding affinities of CDR-grafted anti-CD3 monoclonal antibodies were determined by competition binding (ref. 6) using the HPB-ALL human T cell line as a source of CD3 antigen, and fluorescein-conjugated murine OKT3 (Fl-OKT3) of known binding affinity as a tracer antibody. The binding affinity of Fl-OKT3 tracer antibody was determined by a direct binding assay in which increasing amounts of Fl-OKT3 were incubated with HPB-ALL (5×10^5) in PBS with 5% foetal calf serum for 60 min. at 4°C. Cells were washed, and the fluorescence intensity was determined on a FACScan flow cytometer calibrated with quantitative microbead standards (Flow Cytometry Standards, Research Triangle Park, NC). Fluorescence intensity per antibody molecule (F/P ratio) was determined by using microbeads which have a predetermined number of mouse IgG antibody binding sites (Simply Cellular beads, Flow Cytometry Standards). F/P equals the fluorescence intensity of beads saturated with Fl-OKT3 divided by the number of binding sites per bead. The amount of bound and free Fl-OKT3 was calculated from the mean fluorescence intensity per cell, and the ratio of bound/free was plotted against the number of moles of antibody bound. A linear fit was used to determine the affinity of binding (absolute value of the slope).

For competitive binding, increasing amounts of competitor antibody were added to a sub-saturating dose of Fl-OKT3 and incubated with 5×10^5 HPB-ALL in 200 ml of PBS with 5% foetal calf serum, for 60 min at 4°C. The fluorescence intensities of the cells were measured on a FACScan flow cytometer calibrated with quantitative microbead standards. The concentrations of bound and free Fl-OKT3 were calculated. The affinities of competing anti-bodies were calculated from the equation $[X]-[OKT3] = (1/Kx) - (1/Ka)$, where Ka is the affinity of murine OKT3, Kx is the affinity of competitor X, [ ] is the concentration of competitor antibody at which bound/free binding is R/2, and R is the maximal bound/free binding.

4. cDNA LIBRARY CONSTRUCTION

4.1. mRNA PREPARATION AND cDNA SYNTHESIS

OKT3 producing cells were grown as described above and 1.2×10^9 cells harvested and mRNA extracted using the guanidinium/LiCl extraction procedure. cDNA was prepared by priming from Oligo-dT to generate full length cDNA. The cDNA was methylated and EcoR1 linkers added for cloning.

4.2. LIBRARY CONSTRUCTION

The cDNA library was ligated to pSP65 vector DNA which had been EcoR1 cut and the 5' phosphate groups removed by calf intestinal phosphatase (EcoR1/CIP). The ligation was used to transform high transformation efficiency Escherichia coli (E.coli) HB101. A cDNA library was prepared. 3800 colonies were screened for the light chain and 10000 colonies were screened for the heavy chain.

5. SCREENING

E.coli colonies positive for either heavy or light chain probes were identified by oligonucleotide screening using the oligonucleotides: 5' TCCAGATGTTAACTGCTCAC for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5' CAGGGGCCAGTGGATGGATAGAC for the heavy chain which is complementary to a sequence in the mouse IgG2a constant CH1 domain region. 12

light chain and 9 heavy chain clones were identified and taken for second round screening. Positive clones from the second round of screening were grown up and DNA prepared. The sizes of the gene inserts were estimated by gel electrophoresis and inserts of a size capable of containing a full length cDNA were subcloned into M13 for DNA sequencing.

6. DNA SEQUENCING

Clones representing four size classes for both heavy and light chains were obtained in M13. DNA sequence for the 5' untranslated regions, signal sequences, variable regions and 3' untranslated regions of full length cDNAs [Figures 1(a) and 2(a)] were obtained and the corresponding amino acid sequences predicted [(Figures 1(b) and 2(b)]. In Figure 1(a) the untranslated DNA regions are shown in uppercase, and in both Figures 1 and 2 the signal sequences are underlined.

7. CONSTRUCTION OF cDNA EXPRESSION VECTORS

Celltech expression vectors are based on the plasmid pEE6hCMV (ref. 14). A polylinker for the insertion of genes to be expressed has been introduced after the major immediate early promoter/enhancer of the human Cytomegalovirus (hCMV). Marker genes for selection of the plasmid in transfected eukaryotic cells can be inserted as BamH1 cassettes in the unique BamH1 site of pEE6 hCMV; for instance, the neo marker to provide pEE6 hCMV neo. It is usual practice to insert the neo and gpt markers prior to insertion of the gene of interest, whereas the GS marker is inserted last because of the presence of internal EcoR1 sites in the cassette.

The selectable markers are expressed from the SV40 late promoter which also provides an origin of replication so that the vectors can be used for expression in the COS cell transient expression system.

The mouse sequences were excised from the M13 based vectors described above as EcoR1 fragments and cloned into either pEE6-hCMV-neo for the heavy chain and into EE6-hCMV-gpt for the light chain to yield vectors pJA136 and pJA135 respectively.

8. EXPRESSION OF cDNAS IN COS CELLS

Plasmids pJA135 and pJA136 were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to T-cell enriched lymphocytes. Metabolic labelling experiments using ^{35}S methionine showed expression and assembly of heavy and light chains.

9. CONSTRUCTION OF CHIMERIC GENES

Construction of chimeric genes followed a previously described strategy [Whittle et al (ref. 13)]. A restriction site near the 3' end of the variable domain sequence is identified and used to attach an oligonucleotide adapter coding for the remainder of the mouse variable region and a suitable restriction site for attachment to the constant region of choice.

9.1. LIGHT CHAIN GENE CONSTRUCTION

The mouse light chain cDNA sequence contains an Aval site near the 3' end of the variable region [Fig. 1(a)]. The majority of the sequence of the variable region was isolated as a 396 bp. EcoR1-Aval fragment. An oligonucleotide adapter was designed to replace the remainder of the 3' region of the variable region from the Aval site and to include the 5' residues of the human constant region up to and including a unique Nar1 site which had been previously engineered into the constant region.

A Hind111 site was introduced to act as a marker for insertion of the linker.

The linker was ligated to the V_L fragment and the 413 bp EcoR1-Nar1 adapted fragment was purified from the ligation mixture.

The constant region was isolated as an Nar1-BamH1 fragment from an M13 clone NW361 and was ligated with the variable region DNA into an EcoR1/BamH1/C1P pSP65 treated vector in a three way reaction to yield plasmid JA143. Clones were isolated after transformation into E.coli and the linker and junction sequences were confirmed by the presence of the Hind111 site and by DNA sequencing.

9.2 LIGHT CHAIN GENE CONSTRUCTION - VERSION 2

The construction of the first chimeric light chain gene produces a fusion of mouse and human amino acid sequences at the variable-constant region junction. In the case of the OKT3 light chain the amino acids at the chimera junction are:

........Leu-Glu-Ile-Asn-Arg/ -/Thr-Val-Ala -Ala

VARIABLE **CONSTANT**

This arrangement of sequence introduces a potential site for Asparagine (Asn) linked (N-linked) glycosylation at the V-C junction. Therefore, a second version of the chimeric light chain oligonucleotide adapter was designed in which the threonine (Thr), the first amino acid of the human constant region, was replaced with the equivalent amino acid from the mouse constant region, Alanine (Ala).

An internal Hind111 site was not included in this adapter, to differentiate the two chimeric light chain genes.

The variable region fragment was isolated as a 376 bp EcoR1-Aval fragment. The oligonucleotide linker was ligated to Nar1 cut pNW361 and then the adapted 396bp constant region was isolated after recutting the modified pNW361 with EcoR1. The variable region fragment and the modified constant region fragment were ligated directly into EcoR1/C1P treated pEE6hCMVneo to yield pJA137. Initially all clones examined had the insert in the incorrect orientation. Therefore, the insert was re-isolated and recloned to turn the insert round and yield plasmid pJA141. Several clones with the insert in the correct orientation were obtained and the adapter sequence of one was confirmed by DNA sequencing

9.3. HEAVY CHAIN GENE CONSTRUCTION

9.3.1. CHOICE OF HEAVY CHAIN GENE ISOTYPE

The constant region isotype chosen for the heavy chain was human IgG4.

9.3.2. GENE CONSTRUCTION

The heavy chain cDNA sequence showed a Ban1 site near the 3' end of the variable region [Fig. 2(a)-]. The majority of the sequence of the variable region was isolated as a 426bp. EcoR1/C1P/Ban1 fragment. An oligonucleotide adapter was designated to replace the remainder of the 3' region of the variable region from the Ban1 site up to and including a unique HindIII site which had been previously engineered into the first two amino acids of the constant region.

The linker was ligated to the V_H fragment and the EcoR1-Hind111 adapted fragment was purified from the ligation mixture. The variable region was ligated to the constant region by cutting pJA91 with EcoR1 and Hind111 removing the intron fragment and replacing it with the V_H to yield pJA142. Clones were isolated after transformation into E.coli JM101 and the linker and junction sequences were confirmed by DNA sequencing. (N.B. The Hind111 site is lost on cloning).

10. CONSTRUCTION OF CHIMERIC EXPRESSION VECTORS

10.1. neo AND gpt VECTORS

The chimeric light chain (version 1) was removed from pJA143 as an EcoR1 fragment and cloned into EcoR1/C1P treated pEE6hCMVneo expression vector to yield pJA145. Clones with the insert in the correct orientation were identified by restriction mapping.

The chimeric light chain (version 2) was constructed as described above.

The chimeric heavy chain gene was isolated from pJA142 as a 2.5Kbp EcoR1/BamH1 fragment and cloned into the EcoR1/Bcl1/C1P treated vector fragment of a derivative of pEE6hCMVgpt to yield plasmid pJA144.

10.2. GS SEPARATE VECTORS

GS versions of pJA141 and pJA144 were constructed by replacing the neo and gpt cassettes by a BamH1/Sa11/C1P treatment of the plasmids, isolation of the vector fragment and ligation to a GS-containing fragment from the plasmid pRO49 to yield the light chain vector pJA179 and the heavy chain vector pJA180.

10.3. GS SINGLE VECTOR CONSTRUCTION

Single vector constructions containing the cL (chimeric light), cH (chimeric heavy) and GS genes on one plasmid in the order cL-cH-GS, or cH-cL-GS and with transcription of the genes being head to tail e.g. cL>cH>GS were constructed. These plasmids were made by treating pJA179 or pJA180 with BamH1/C1P and ligating in a Bgl11/Hind111 hCMV promoter cassette along with either the Hind111/BamH1 fragment from pJA141 into pJA180 to give the cH-cL-GS plasmid pJA182 or the Hind111/BamH1 fragment from pJA144 into pJA179 to give the cL-cH-GS plasmid pJA181.

11. EXPRESSION OF CHIMERIC GENES

11.1. EXPRESSION IN COS CELLS

The chimeric antibody plasmid pJA145 (cL) and pJA144 (cH) were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to the HUT 78 human T-cell line. Metabolic labelling experiments using ^{35}S methionine showed expression and assembly of heavy and light chains. However the light chain mobility seen on reduced gels suggested that the potential glycosylation site was being glycosylated. Expression in COS cells in the presence of tunicamycin showed a reduction in size of the light chain to that shown for control chimeric antibodies and the OKT3 mouse light chain. Therefore JA141 was constructed and expressed. In this case the light chain did not show an aberrant mobility or a size shift in the presence or absence of tunicamycin. This second version of the chimeric light chain, when expressed in association with chimeric heavy (cH) chain, produced antibody which showed good binding to HUT

78 cells. In both cases antigen binding was equivalent to that of the mouse antibody.

11.2 EXPRESSION IN CHINESE HAMSTER OVARY (CHO) CELLS Stable cell lines have been prepared from plasmids PJA141/pJA144 and from pJA179/pJA180, pJA181 and pJA182 by transfection into CHO cells.

12. CDR-GRAFTING

The approach taken was to try to introduce sufficient mouse residues into a human variable region framework to generate antigen binding activity comparable to the mouse and chimeric antibodies.

12.1. VARIABLE REGION ANALYSIS

From an examination of a small database of structures of antibodies and antigen-antibody complexes it is clear that only a small number of antibody residues make direct contact with antigen. Other residues may contribute to antigen binding by positioning the contact residues in favourable configurations and also by inducing a stable packing of the individual variable domains and stable interaction of the light and heavy chain variable domains.

The residues chosen for transfer can be identified in a number of ways:

(a) By examination of antibody X-ray crystal structures the antigen binding surface can be predominantly located on a series of loops, three per domain, which extend from the B-barrel framework.

(b) By analysis of antibody variable domain sequences regions of hypervariability [termed the Complementarity Determining Regions (CDRs) by Wu and Kabat (ref. 5)] can be identified. In the most but not all cases these CDRs correspond to, but extend a short way beyond, the loop regions noted above.

(c) Residues not identified by (a) and (b) may contribute to antigen binding directly or indirectly by affecting antigen binding site topology, or by inducing a stable packing of the individual variable domains and stabilising the inter-variable domain interaction. These residues may be identified either by superimposing the sequences for a given antibody on a known structure and looking at key residues for their contribution, or by sequence alignment analysis and noting "idiosyncratic" residues followed by examination of their structural location and likely effects.

12.1.1. LIGHT CHAIN

Figure 3 shows an alignment of sequences for the human framework region RE1 and the OKT3 light variable region. The structural loops (LOOP) and CDRs (KABAT) believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 13.1(c). Above the sequence in Figure 3 the residue type indicates the spatial location of each residue side chain, derived by examination of resolved structures from X-ray crystallography analysis. The key to this residue type designation is as follows:

N - near to CDR (From X-ray Structures)
P - Packing B - Buried Non-Packing
S - Surface E - Exposed
I - Interface * - Interface
- Packing/Part Exposed
? - Non-CDR Residues which may require to be left as Mouse sequence. Residues underlined in Figure 3 are amino acids. RE1 was chosen as the human framework because the light chain is a kappa chain and the kappa variable regions show higher homology with the mouse sequences than a lambda light variable region, e.g. KOL (see below). RE1 was chosen in preference to another kappa light chain because the X-ray structure of the light chain has been determined so that a structural examination of individual residues could be made.

12.1.2. HEAVY CHAIN

Similarly Figure 4 shows an alignment of sequences for the human framework region KOL and the OKT3 heavy variable region. The structural loops and CDRs believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 12.1(c). The residue type key and other indicators used in Figure 4 are the same as those used in Figure 3. KOL was chosen as the heavy chain framework because the X-ray structure has been determined to a better resolution than, for example, NEWM and also the sequence alignment of OKT3 heavy variable region showed a slightly better homology to KOL than to NEWM.

12.2. DESIGN OF VARIABLE GENES

The variable region domains were designed with mouse variable region optimal codon usage [Grantham and Perrin (ref. 15)] and used the B72.3 signal sequences [Whittle et al (ref. 13)]. The sequences were designed to be attached to the constant region in the same way as for the chimeric genes described above. Some constructs contained the "Kozak consensus sequence" [Kozak (ref. 16)] directly linked to the 5' of the signal sequence in the gene. This sequence motif is believed to have a beneficial role in translation initiation in eukaryotes.

12.3. GENE CONSTRUCTION

To build the variable regions, various strategies are available. The sequence may be assembled by using oligonucleotides in a manner similar to Jones et al (ref. 17) or by simultaneously replacing all of the CDRs or loop regions by oligonucleotide directed site specific mutagenesis in a manner similar to Verhoeyen et al (ref. 2). Both strategies were used and a list of constructions is set out in Tables 1 and 2 and Figures 4 and 5. It was noted in several cases that the mutagenesis approach led to deletions and rearrangements in the gene being remodelled, while the success of the assembly approach was very sensitive to the quality of the oligonucleotides.

13. CONSTRUCTION OF EXPRESSION VECTORS

Genes were isolated from M13 or SP65 based intermediate vectors and cloned into pEE6hCMVneo for the light chains and pEE6hCMVgpt for the heavy chains in a manner similar to that for the chimeric genes as described above.

TABLE 1 CDR-GRAFTED GENE CONSTRUCTS

CODE	MOUSE SEQUENCE CONTENT	METHOD OF CONSTRUCTION	KOZAK SEQUENCE	
			-	+
LIGHT CHAIN	ALL HUMAN FRAMEWORK RE1			
121	26-32, 50-56, 91-96 inclusive	SDM and gene assembly	+	n.d.
121A	26-32, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	n.d.	+
121B	26-32, 50-56, 91-96 inclusive + 46, 47	Partial gene assembly	n.d.	+
221	24-24, 50-56, 91-96 inclusive	Partial gene assembly	+	+
221A	24-34, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	+	+
221B	24-34, 50-56, 91-96 inclusive +1, 3	Partial gene assembly	+	+
221C	24-34, 50-56, 91-96 inclusive	Partial gene assembly	+	+
HEAVY CHAIN	ALL HUMAN FRAMEWORK KOL			
121	26-32, 50-56, 95-100B inclusive	Gene assembly	n.d.	+
131	26-32, 50-58, 95-100B inclusive	Gene assembly	n.d.	+
141	26-32, 50-65, 95-100B inclusive	Partial gene assembly	+	n.d.
321	26-35, 50-56, 95-100B inclusive	Partial gene assembly	+	n.d.
331	26-35, 50-58, 95-100B inclusive	Partial gene assembly	+	
		Gene assembly		+
341	26-35, 50-65, 95-100B inclusive	SDM	+	
		Partial gene assembly		+
341A	26-35, 50-65, 95-100B inclusive +6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91 (+63 = human)	Gene assembly	n.d.	+
341B	26-35, 50-65, 95-100B inclusive + 48, 49, 71, 73, 76, 78, 88, 91 (+63 + human)	Gene assembly	n.d.	+

KEY

n.d.	not done
SDM	Site directed mutagenesis
Gene assembly	Variable region assembled entirely from oligonucleotides
Partial gene assembly	Variable region assembled by combination of restriction fragments either from other genes originally created by SDM and gene assembly or by oligonucleotide assembly of part of the variable region and reconstruction with restriction fragments from other genes originally created by SDM and gene assembly

14. EXPRESSION OF CDR-GRAFTED GENES

14.1. PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED LIGHT (gL) CHAINS WITH MOUSE HEAVY (mH) OR CHIMERIC HEAVY (cH) CHAINS

All gL chains, in association with mH or cH produced reasonable amounts of antibody. Insertion of the Kozak consensus sequence at a position 5' to the ATG (kgL constructs) however, led to a 2-5 fold improvement in net expression. Over an extended series of experiments expression levels were raised from approximately 200ng/ml to approximately 500 ng/ml for kgL/cH or kgL/mH combinations.

When direct binding to antigen on HUT 78 cells was measured, a construct designed to include mouse sequence based on loop length (gL121) did not lead to active antibody in association with mH or cH. A construct designed to include mouse sequence based on Kabat CDRs (gL221) demonstrated some weak binding in association with mH or cH. However, when framework residues 1, 3, 46, 47 were changed from the human to the murine OKT3 equivalents based on the arguments outlined in Section 12.1 antigen binding was demonstrated when both of the new constructs, which were termed 121A and 221A were co-expressed with cH. When the effects of these residues were examined in more detail, it appears that residues 1 and 3 are not major contributing residues as the product of the gL221B gene shows little detectable binding activity in association with cH. The light chain product of gL221C, in which mouse sequences are present at 46 and 47, shows good binding activity in association with cH.

14.2 PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED HEAVY (gH) CHAINS WITH MOUSE LIGHT (mL) OR CHIMERIC LIGHT (cL) CHAINS

Expression of the gH genes proved to be more difficult to achieve than for gL. First, inclusion of the Kozak sequence appeared to have no marked effect on expression of gH genes. Expression appears to be slightly improved but not to the same degree as seen for the grafted light chain.

Also, it proved difficult to demonstrate production of expected quantities of material when the loop choice (amino acid 26-32) for CDR1 is used, e.g. gH121, 131, 141 and no conclusions can be drawn about these constructs.

Moreover, co-expression of the gH341 gene with cL or mL has been variable and has tended to produce lower amounts of antibody than the cH/cL or mH/mL combinations. The alterations to gH341 to produce gH341A and gH341B lead to improved levels of expression.

This may be due either to a general increase in the fraction of mouse sequence in the variable region, or to the alteration at position 63 where the residue is returned to the human amino acid Valine (Val) from Phenylalanine (Phe) to avoid possible internal packing problems with the rest of the human framework. This arrangement also occurs in gH331 and gH321.

When gH321 or gH331 were expressed in association with cL, antibody was produced but antibody binding activity was not detected.

When the more conservative gH341 gene was used antigen binding could be detected in association with cL or mL, but the activity was only marginally above the background level. When further mouse residues were substituted based on the arguments in 12.1, antigen binding could be clearly demonstrated for the antibody produced when kgH341A and kgH341B were expressed in association with cL.

14.3 PRODUCTION OF FULLY CDR-GRAFTED ANTIBODY

The kgL221A gene was co-expressed with kgH341, kgH341A or kgH341B. For the combination kgH221A/kgH341 very little material was produced in a normal COS cell expression.

For the combinations kgL221A/kgH341A or kgH221A/kgH341B amounts of antibody similar to gL/cH was produced.

In several experiments no antigen binding activity could be detected with kgH221A/gH341 or kgH221A/kgH341 combinations, although expression levels were very low.

Antigen binding was detected when kgL221A/kgH341A or kgH221A/kgH341B combinations were expressed. In the case of the antibody produced from the kgL221A/kgH341A combination the antigen binding was very similar to that of the chimeric antibody.

An analysis of the above results is given below.

15. DISCUSSION OF CDR-GRAFTING RESULTS

In the design of the fully humanised antibody the aim was to transfer the minimum number of mouse amino acids that would confer antigen binding onto a human antibody framework.

15.1. LIGHT CHAIN

15.1.1. EXTENT OF THE CDRs

For the light chain the regions defining the loops known from structural studies of other antibodies to contain the antigen contacting residues, and those hypervariable sequences defined by Kabat et al (refs. 4 and 5) as Complementarity Determining Regions (CDRs) are equivalent for CDR2. For CDR1 the hypervariable region extends from residues 24-34 inclusive while the structural loop extends from 26-32 inclusive. In the case of OKT3 there is only one amino acid difference between the two options, at amino acid 24, where the mouse sequence is a serine and the human framework RE1 has glutamine. For CDR3 the loop extends from residues 91-96 inclusive while the

Kabat hypervariability extends from residues 89-97 inclusive. For OKT3 amino acids 89, 90 and 97 are the same between OKT3 and RE1 (Fig. 3). When constructs based on the loop choice for CDR1 (gL121) and the Kabat choice (gL221) were made and co-expressed with mH or cH no evidence for antigen binding activity could be found for gL121, but trace activity could be detected for the gL221, suggesting that a single extra mouse residue in the grafted variable region could have some detectable effect. Both gene constructs were reasonably well expressed in the transient expression system.

15.1.2. FRAMEWORK RESIDUES

The remaining framework residues were then further examined, in particular amino acids known from X-ray analysis of other antibodies to be close to the CDRs and also those amino acids which in OKT3 showed differences from the consensus framework for the mouse subgroup (subgroup VI) to which OKT3 shows most homology. Four positions 1, 3, 46 and 47 were identified and their possible contribution was examined by substituting the mouse amino acid for the human amino acid at each position. Therefore gL221A (gL221 + D1Q, Q3V, L46R, L47W, see Figure 3 and Table 1) was made, cloned in EE6hCMVneo and co-expressed with cH (pJA144). The resultant antibody was well expressed and showed good binding activity. When the related genes gL221B (gL221 + D1Q, Q3V) and gL221C (gL221 + L46R, L47W) were made and similarly tested, while both genes produced antibody when co-expressed with cH, only the gL221C/cH combination showed good antigen binding. When the gL121A (gL121 + D1Q, Q3V, L46R, L47W) gene was made and co-expressed with cH, antibody was produced which also bound to antigen.

15.2. HEAVY CHAIN

15.2.1. EXTENT OF THE CDRs

For the heavy chain the loop and hypervariability analyses agree only in CDR3. For CDR1 the loop region extends from residues 26-32 inclusive whereas the Kabat CDR extends from residues 31-35 inclusive. For CDR2 the loop region is from 50-58 inclusive while the hypervariable region covers amino acids 50-65 inclusive. Therefore humanised heavy chains were constructed using the framework from antibody KOL and with various combinations of these CDR choices, including a shorter choice for CDR2 of 50-56 inclusive as there was some uncertainty as to the definition of the end point for the CDR2 loop around residues 56 to 58. The genes were co-expressed with mL or cL initially. In the case of the gH genes with loop choices for CDR1 e.g. gH121, gH131, gH141 very little antibody was produced in the culture supernatants. As no free light chain was detected it was presumed that the antibody was being made and assembled inside the cell but that the heavy chain was aberrant in some way, possibly incorrectly folded, and therefore the antibody was being degraded internally. In some experiments trace amounts of antibody could be detected in ^{35}S labelling studies.

As no net antibody was produced, analysis of these constructs was not pursued further.

When, however, a combination of the loop choice and the Kabat choice for CDR1 was tested (mouse amino acids 26-35 inclusive) and in which residues 31 (Ser to Arg), 33 (Ala to Thr), and 35 (Tyr to His) were changed from the human residues to the mouse residue and compared to the first series, antibody was produced for gH321, kgH331 and kgH341 when co-expressed with cL. Expression was generally low and could not be markedly improved by the insertion of the Kozak consensus sequence 5' to the ATG of the signal sequence of the gene, as distinct from the case of the gL genes where such insertion led to a 2-5 fold increase in net antibody production. However, only in the case of gH341/mL or kgH341/cL could marginal antigen binding activity be demonstrated. When the kgH341 gene was co-expressed with kgL221A, the net yield of antibody was too low to give a signal above the background level in the antigen binding assay.

15.2.2. FRAMEWORK RESIDUES

As in the case of the light chain the heavy chain frameworks were re-examined. Possibly because of the lower initial homology between the mouse and human heavy variable domains compared to the light chains, more amino acid positions proved to be of interest. Two genes kgH341A and kgH341B were constructed, with 11 or 8 human residues respectively substituted by mouse residues compared to gH341, and with the CDR2 residue 63 returned to the human amino acid potentially to improve domain packing. Both showed antigen binding when combined with cL or kgL221A, the kgH341A gene with all 11 changes appearing to be the superior choice.

15.3 INTERIM CONCLUSIONS

It has been demonstrated, therefore, for OKT3 that to transfer antigen binding ability to the humanised antibody, mouse residues outside the CDR regions defined by the Kabat hypervariability or structural loop choices are required for both the light and heavy chains. Fewer extra residues are needed for the light chain, possibly due to the higher initial homology between the mouse and human kappa variable regions.

Of the changes seven (1 and 3 from the light chain and 6, 23, 71, 73 and 76 from the heavy chain) are predicted from a knowledge of other antibody structures to be either partly exposed or on the antibody surface. It has been shown here that residues 1 and 3 in the light chain are not absolutely required to be the mouse sequence; and for the heavy chain the gH341B heavy chain in combination with the 221A light chain generated only weak binding activity. Therefore the presence of the 6, 23 and 24 changes are important to maintain a binding affinity similar to that of the murine antibody. It was important, therefore, to further study the individual contribution of othe other 8 mouse residues of the kgH341A gene compared to kgH341.

16. FURTHER CDR-GRAFTING EXPERIMENTS

Additional CDR-grafted heavy chain genes were prepared substantially as described above. With reference to Table 2 the further heavy chain genes were based upon the gh341 (plasmid pJA178) and gH341A (plasmid pJA185) with either mouse OKT3 or human KOL residues at 6, 23, 24, 48, 49, 63, 71, 73, 76, 78, 88 and 91, as indicated. The CDR-grafted light chain genes used in these further experiments were gL221, gL221A, gL221B and gL221C as described above.

TABLE 2

OKT3 HEAVY CHAIN CDR GRAFTS

1. gH341 and derivatives

RES NUM	6	23	24	48	49	63	71	73	76	78	88	91	
OKT3vh	Q	K	A	I	G	F	T	K	S	A	A	Y	
gH341	E	S	S	V	A	F	R	N	N	L	G	F	JA178
gH341A	Q	K	A	I	G	V	T	K	S	A	A	Y	JA185
gH341E	Q	K	A	I	G	V	T	K	S	A	G	G	JA198
gH341*	Q	K	A	I	G	V	T	K	N	A	G	F	JA207
gH341*	Q	K	A	I	G	V	R	N	N	A	G	F	JA209
gH341D	Q	K	A	I	G	V	T	K	N	L	G	F	JA197
gH341*	Q	K	A	I	G	V	R	N	N	L	G	F	JA199
gH341C	Q	K	A	V	A	F	R	N	N	L	G	F	JA184
gH341*	Q	S	A	I	G	V	T	K	S	A	A	Y	JA203
gH341*	E	S	A	I	G	V	T	K	S	A	A	Y	JA205
gH341B	E	S	S	I	G	V	T	K	S	A	A	Y	JA183
gH341*	Q	S	A	I	G	V	T	K	S	A	G	F	JA204
gH341*	E	S	A	I	G	V	T	K	S	A	G	F	JA206
gH341*	Q	S	A	I	G	V	T	K	N	A	G	F	JA208
KOL	E	S	S	V	A		R	N	N	L	G	F	

OKT3 LIGHT CHAIN CDR GRAFTS

2. gL221 and derivatives

RES NUM	1	3	46	47	
OKT3vl	Q	V	R	W	
CL221	D	Q	L	L	DA221
gL221A	Q	V	R	W	DA221A
gL221B	Q	V	L	L	DA221B
CL221C	D	Q	R	W	DA221C
RE1	D	Q	L	L	

MURINE RESIDUES ARE UNDERLINED

The CDR-grafted heavy and light chain genes were co-expressed in COS cells either with one another in various combinations but also with the corresponding murine and chimeric heavy and light chain genes substantially as described above. The resultant antibody products were then assayed in binding and blocking assays with HPB-ALL cells as described above.

The results of the assays for various grafted heavy chains co-expressed with the gL221C light chain are given in Figures 7 and 8 (for the JA184, JA185, JA197 and JA198 constructs - see Table 2), in Figure 9 (for the JA183, JA184, JA185 and JA197 constructs) in Figure 10 (for the chimeric, JA185, JA199, JA204, JA205, JA207, JA208 and JA209 constructs) and in Figure 11 (for the JA183, JA184, JA185, JA198, JA203, JA205 and JA206 constructs).

The basic grafted product without any human to murine changes in the variable frameworks, i.e. gL221 co-expressed with gh341 (JA178), and also the "fully grafted" product, having most human to murine changes in the grafted heavy chain framework, i.e. gL221C co-expressed with gh341A (JA185), were assayed for relative binding affinity in a competition assay against murine OKT3 reference standard, using HPB-ALL cells. The assay used was as described above in section 3.3. The results obtained are given in Figure 12 for the basic grafted product and in Figure 13 for the fully grafted product. These results indicate that the basic grafted product has neglibible binding ability as compared with the OKT3 murine reference standard; whereas the "fully grafted" product has a binding ability very similar to that of the OKT3 murine reference standard.

The binding and blocking assay results indicate the following:
The JA198 and JA207 constructs appear to have the best binding characteristics and similar binding abilities, both substantially the same as the chimeric and fully grafted gH341A products. This indicates that positions 88 and 91 and position 76 are not highly critical for maintaining the OKT3 binding ability; whereas at least some of positions 6, 23, 24, 48, 49, 71, 73 and 78 are more important.

This is borne out by the finding that the JA209 and JA199, although of similar binding ability to one another, are of lower binding ability than the JA198 and JA207 constructs. This indicates the importance of having mouse residues at positions 71, 73 and 78, which are either completely or partially human in the JA199 and JA209 constructs respectively.

Moreover, on comparing the results obtained for the JA205 and JA183 constructs it is seen that there is a decrease in binding going from the JA205 to the JA183 constructs. This indicates the importance of retaining a mouse residue at position 23, the only position changed between JA205 and JA183.

These and other results lead us to the conclusion that of the 11 mouse framework residues used in the gH341A (JA185) construct, it is important to retain mouse residues at all of positions 6, 23, 24, 48 and 49, and possibly for maximum binding affinity at 71, 73 and 78.

Similar Experiments were carried out to CDR-graft a number of the rodent antibodies including antibodies having specificity for CD4 (OKT4), ICAM-1 (R6-5), TAG72 (B72.3), and TNFα(61E71, 101.4, hTNF1, hTNF2 and hTNF3).

EXAMPLE 2

CDR-GRAFTING OF A MURINE ANTI-CD4 T CELL RECEPTOR ANTIBODY, OKT4A

Anti OKT4A CDR-grafted heavy and light chain genes were prepared, expressed and tested substantially as described above in Example 1 for CDR-grafted OKT3. The CDR grafting of OKT4A is described in detail in Ortho patent application PCT/GB 90......... of even date herewith entitled "Humanised Antibodies". The disclosure of this Ortho patent application PCT/GB 90 .... is incorporated herein by reference. A number of CDR-grafted OKT4 antibodies have been prepared. Presently the CDR-grafted OKT4A of choice is the combination of the grafted light chain LCDR2 and the grafted heavy chain HCDR10.

THE LIGHT CHAIN

The human acceptor framework used for the grafted light chains was RE1. The preferred LCDR2 light chain has human to mouse changes at positions 33, 34, 38, 49 and 89 in addition to the structural loop CDRs. Of these changed positions, positions 33, 34 and 89 fall within the preferred extended CDRs of the present invention (positions 33 and 34 in CDR1 and position 89 in CDR3).
The human to murine changes at positions 38 and 49 corresponds to positions at which the amino acid residues are preferably donor murine amino acid residues in accordance with the present invention.
A comparison of the amino acid sequences of the donor murine light chain variable domain and the RE1 human acceptor light chain variable further reveals that the murine and human residues are identical at all of positions 46, 48 and 71 and at all of positions 2, 4, 6, 35, 36, 44, 47, 62, 64-69, 85, 87, 98, 99 and 101 and 102. However the amino acid residue at position 58 in LCDR2 is the human RE1 framework residue not the mouse OKT4 residue as would be preferred in accordance with the present invention.

THE HEAVY CHAIN

The human acceptor framework used for the grafted heavy chains was KOL.
The preferred CDR graft HCDR10 heavy chain has human to mouse changes at positions 24, 35, 57, 58, 60, 88 and 91 in addition to the structural loop CDRs.
Of these positions, positions 35 (CDR1) and positions 57, 58 and 60 (CDR2) fall within the preferred extended CDRs of the present invention. Also the human to mouse change at position 24 corresponds to a position at which the amino acid residue is a donor murine residue in accordance with the present invention. Moreover, the human to mouse changes at positions 88 and 91 correspond to positions at which the amino acid residues are optionally donor murine residues.
Moreover, a comparison of the murine OKT4A and human KOL heavy chain variable amino acid sequences reveals that the murine and human residues are identical at all of positions 23, 49, 71, 73 and 78 and at all of positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.
Thus the OKT4A CDR-grafted heavy chain HCDR10 corresponds to a particularly preferred embodiment according to the present invention.

EXAMPLE 3

CDR-GRAFTING OF AN ANTI-MUCIN SPECIFIC MURINE ANTIBODY, B72.3

The cloning of the genes coding for the anti-mucin specific murine monoclonal antibody B72.3 and the preparation of B72.3 mouse-human chimeric antibodies has been described previously (ref. 13 and WO 89/01783). CDR-grafted versions of B72.3 were prepared as follows.
(a) B72.3 Light Chain
CDR-grafting of this light chain was accomplished by direct transfer of the murine CDRs into the framework of the human light chain RE1. The regions transferred were:

CDR Number	Residues
1	24-34
2	50-56
3	90-96

The activity of the resulting grafted light chain was assessed by co-expression in COS cells, of genes for the combinations:
B72.3 cH/B72.3 cL
and B72.3 cH/B72.3 gL
Supernatants were assayed for antibody concentration and for the ability to bind to microtitre plates coated with mucin. The results obtained indicated that, in combination with the B72.3 cH chain, B72.3 cL and B72.3 gL had similar binding properties.
Comparison of the murine B72.3 and REI light chain amino acid sequences reveals that the residues are identical at positions 46, 58 and 71 but are different at position 48. Thus changing the human residue to the donor mouse residue at position 48 may further improve the binding characteristics of the CDR-grafted Light chain, (B72.3 gL) in accordance with the present invention.
(b) B72.3 heavy chain
i. Choice of framework
At the outset it was necessary to make a choice of human framework. Simply put, the question was as follows: Was it necessary to use the framework regions from an antibody whose crystal structure was known or could the choice be made on some other criteria?
For B72.3 heavy chain, it was reasoned that, while knowledge of structure was important, transfer of the CDRs from mouse to human frameworks might be facilitated if the overall homology between the donor and receptor frameworks was maximised. Comparison of the B72.3 heavy chain sequence with those in Kabat (ref. 4) for human heavy chains showed clearly that B72.3 had poor homology for KOL and NEWM (for which crystal structures are available) but was very homologous to the heavy chain for EU.
On this basis, EU was chosen for the CDR-grafting and the following residues transferred as CDRs.

CDR Number	Residues
1	27-36
2	50-63
3	93-102

Also it was noticed that the FR4 region of EU was unlike that of any other human (or mouse) antibody. Consequently, in the grafted heavy chain genes this was also changed to produce a "consensus" human sequence. (Preliminary experiments showed that grafted heavy chain genes containing the EU FR4 sequence expressed very poorly in transient expression systems.)

ii. Results with grafted heavy chain genes

Expression of grafted heavy chain genes containing all human framework regions with either gL or cL genes produced a grafted antibody with little ability to bind to mucin. The grafted antibody had about 1% the activity of the chimeric antibody. In these experiments, however, it was noted that the activity of the grafted antibody could be increased to ~ 10% of B72.3 by exposure to pHs of 2-3.5.

This observation provided a clue as to how the activity of the grafted antibody could be improved without acid treatment. It was postulated that acid exposure brought about the protonation of an acidic residue (pKa of aspartic acid = 3.86 and of glutamine acid = 4.25) which in turn caused a change in structure of the CDR loops, or allowed better access of antigen.

From comparison of the sequences of B72.3 (ref. 13) and EU (refs. 4 and 5), it was clear that, in going from the mouse to human frameworks, only two positions had been changed in such a way that acidic residues had been introduced. These positions are at residues 73 and 81, where K to E and Q to E changes had been made, respectively.

Which of these positions might be important was determined by examining the crystal structure of the KOL antibody. In KOL heavy chain, position 81 is far removed from either of the CDR loops.

Position 73, however, is close to both CDRs 1 and 3 of the heavy chain and, in this position it was possible to envisage that a K to E change in this region could have a detrimental effect on antigen binding.

iii. Framework changes in B72.3 gH gene

On the basis of the above analysis, E73 was mutated to a lysine (K). It was found that this change had a dramatic effect on the ability of the grafted Ab to bind to mucin. Further the ability of the grafted B72.3 produced by the mutated gH/gL combination to bind to mucin was similar to that of the B72.3 chimeric antibody.

iv. Other framework changes

In the course of the above experiments, other changes were made in the heavy chain framework regions. Within the accuracy of the assays used, none of the changes, either alone or together, appeared beneficial.

v. Other

All assays used measured the ability of the grafted Ab to bind to mucin and, as a whole, indicated that the single framework change at position 73 is sufficient to generate an antibody with similar binding properties to B72.3.

Comparison of the B72.3 murine and EU heavy chain sequences reveals that the mouse and human residues are identical at positions 23, 24, 71 and 78.

Thus the mutated CDR-grafted B72.3 heavy chain corresponds to a preferred embodiment of the present invention.

EXAMPLE 4

CDR-GRAFTING OF A MURINE ANTI-ICAM-1 MONOCLONAL ANTIBODY

A murine antibody, R6-5-D6 (EP 0314863) having specificity for Intercellular Adhesion Molecule 1 (ICAM-1) was CDR-grafted substantially as described above in previous examples. This work is described in greater detail in co-pending application, British Patent Application No. 9009549.8, the disclosure of which is incorporated herein by reference.

The human EU framework was used as the acceptor framework for both heavy and light chains. The CDR-grafted antibody currently of choice is provided by co-expression of grafted light chain gL221A and grafted heavy chain gH341D which has a binding affinity for ICAM 1 of about 75% of that of the corresponding mouse-human chimeric antibody.

LIGHT CHAIN

..gL221A has murine CDRs at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition several framework residues are also the murine amino acid. These residues were chosen after consideration of the possible contribution of these residues to domain packing and stability of the conformation of the antigen binding region. The residues which have been retained as mouse are at positions 2, 3, 48 (?), 60, 84, 85 and 87. Comparison of the murine anti-ICAM 1 and human EU light chain amino acid sequences reveals that the murine and human residues are identical at positions 46, 58 and 71.

HEAVY CHAIN

gH341D has murine CDRs at positions 26-35 (CDR1), 50-56 (CDR2) and94-100B (CDR3). In addition murine residues were used in gH341D at positions 24, 48, 69, 71, 73, 80, 88 and 91. Comparison of the murine anti-ICAM 1 and human EU heavy chain amino acid sequences are identical at positions 23, 49 and 78.

EXAMPLE 5

CDR-Grafting of murine anti-TNFα antibodies

A number of murine anti-TNFα monoclonal antibodies were CDR-grafted substantially as described above in previous examples. These antibodies include the murine monoclonal antibodies designated 61 E71, hTNF1, hTNF3 and 101.4 A brief summary of the CDR-grafting of each of these antibodies is given below.

61E71

A similar analysis as described above (Example 1, Section 12.1.) was done for 61E71 and for the heavy chain 10 residues were identified at 23, 24, 48, 49, 68, 69, 71, 73, 75 and 88 as residues to potentially retain as murine. The human frameworks chosen for CDR-grafting of this antibody, and the hTNF3 and 101.4 antibodies were RE1 for the light chain and KOL for the heavy chain.
Three genes were built, the first of which contained 23, 24, 48, 49, 71 and 73 [gH341(6)] as murine residues. The second gene also had 75 and 88 as murine residues [gH341(8)] while the third gene additionally had 68, 69, 75 and 88 as murine residues [gH341(10)]. Each was co-expressed with gL221, the minimum grafted light chain (CDRs only). The gL221/gH341(6) and gL221/gH341(8) antibodies both bound as well to TNF as murine 61E71. The gL221/gH341(10) antibody did not express and this combination was not taken further.
Subsequently the gL221/gH341(6) antibody was assessed in an L929 cell competition assay in which the antibody competes against the TNF receptor on L929 cells for binding to TNF in solution. In this assay the gL221/gH341(6) antibody was approximately 10% as active as murine 61E71.

hTNF1

hTNF1 is a monoclonal antibody which recognises an epitope on human TNF- . The EU human framework was used for CDR-grafting of both the heavy and light variable domains.

Heavy Chain

In the CDR-grafted heavy chain (ghTNF1) mouse CDRs were used at positions 26-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3). Mouse residues were also used in the frameworks at positions 48, 67, 69, 71, 73, 76, 89, 91, 94 and 108. Comparison of the TNF1 mouse and EU human heavy chain residues reveals that these are identical at positions 23, 24, 29 and 78.

Light Chain

In the CDR-grafted light chain (gLhTNF1) mouse CDRs wre used at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition mouse residues were used in the frameworks at positions 3, 42, 48, 49, 83, 106 and 108. Comparison of the hTNF1 mouse and EU human light chain residues reveals that these are identical at positions 46, 58 and 71.

The grafted hTNF1 heavy chain was co-expressed with the chimeric light chain and the binding ability of the product compared with that of the chimeric light chain/chimeric heavy chain product in a TNF binding assay. The grafted heavy chain product appeared to have binding ability for TNF slightly better than the fully chimeric product.

Similarly, a grafted heavy chain/grafted light chain product was co-expressed and compared with the fully chimeric product and found to have closely similar binding properties to the latter product.

hTNF3

hTNF3 recognises an epitope on human TNF-α. The sequence of hTNF3 shows only 21 differences compared to 61E71 in the light and heavy chain variable regions, 10 in the light chain (2 in the CDRs at positions 50, 96 and 8 in the framework at 1, 19, 40, 45, 46, 76, 103 and 106) and 11 in the heavy chain (3 in the CDR regions at positions 52, 60 and 95 and 8 in the framework at 1, 10, 38, 40, 67, 73, 87 and 105). The light and heavy chains of the 61E71 and hTNF3 chimeric antibodies can be exchanged without loss of activity in the direct binding assay. However 61E71 is an order of magnitude less able to compete with the TNF receptor on L929 cello for TNF-a compared to hTNF3. Based on the 61E71 CDR grafting data gL221 and gH341(+23, 24, 48, 49 71 and 73 as mouse) genes have been built for hTNF3 and tested and the resultant grafted antibody binds well to TNF-a, but competes very poorly in the L929 assay. It is possible that in this case also the framework residues identified for OKT3 programme may improve the competitive binding ability of this antibody.

101.4

101.4 is a further murine monoclonal antibody able to recognise human TNF-a. The heavy chain of this antibody shows good homology to KOL and so the CDR-grafting has been based on RE1 for the light chain and KOL for the heavy chain. Several grafted heavy chain genes have been constructed with conservative choices for the CDR's (gH341) and which have one or a small number of non-CDR residues at positions 73, 78 or 77-79 inclusive, as the mouse amino acids. These have been co-expressed with cL or gL221. In all cases binding to TNF equivalent to the chimeric antibody is seen and when co-expressed with cL the resultant antibodies are able to compete well in the L929 assay. However, with gL221 the resultant antibodies are at least an order of magnitude less able to compete for TNF against the TNF receptor on L929 cells.

Mouse residues at other positions in the heavy chain, for example, at 23 and 24 together or at 76 have been demonstrated to provide no improvement to the competitive ability of the grafted antibody in the L929 assay.

A number of other antibodies including antibodies having specificity for interleukins e.g. IL1 and cancer markers such as carcinoembryonic antigen (CEA) e.g. the monoclonal antibody A5B7 (ref. 21), have been successfully CDR-grafted according to the present invention.

It will be appreciated that the foregoing examples are given by way of illustration only and are not intended to limit the scope of the claimed invention. Changes and modifications may be made to the methods described whilst still falling within the spirit and scope of the invention.

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```
                         SEQUENCE LISTING

(1) GENERAL INFORMATION:

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   (ii) TITLE OF INVENTION: HUMANISED ANTIBODIES

  (iii) NUMBER OF SEQUENCES: 33

   (iv) COMPUTER READABLE FORM:
         (A) MEDIUM TYPE: Floppy disk
         (B) COMPUTER: IBM PC compatible
         (C) OPERATING SYSTEM: PC-DOS/MS-DOS
         (D)  SOFTWARE:  PatentIn  Release  #1.0,  Version  #1.25
(EPO)


(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 20 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCCAGATGTT AACTGCTCAC
       20

(2) INFORMATION FOR SEQ ID NO: 2:

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         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAGGGGCCAG TGGATGGATA GAC
       23

(2) INFORMATION FOR SEQ ID NO: 3:

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         (A) LENGTH: 9 amino acids
         (B) TYPE: amino acid
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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Leu Glu Ile Asn Arg Thr Val Ala Ala
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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 943 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAATTCCCAA AGACAAAATG GATTTTCAAG TGCAGATTTT CAGCTTCCTG CTAATCAGTG
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CCTCAGTCAT AATATCCAGA GGACAAATTG TTCTCACCCA GTCTCCAGCA ATCATGTCTG
120

CATCTCCAGG GGAGAAGGTC ACCATGACCT GCAGTGCCAG CTCAAGTGTA AGTTACATGA
180

ACTGGTACCA GCAGAAGTCA GGCACCTCCC CCAAAAGATG GATTTATGAC ACATCCAAAC
240

TGGCTTCTGG AGTCCCTGCT CACTTCAGGG GCAGTGGGTC TGGGACCTCT TACTCTCTCA
300

CAATCAGCGG CATGGAGGCT GAAGATGCTG CCACTTATTA CTGCCAGCAG TGGAGTAGTA
360

ACCCATTCAC GTTCGGCTCG GGGACAAAGT TGGAAATAAA CCGGGCTGAT ACTGCACCAA
420

CTGTATCCAT CTTCCCACCA TCCAGTGAGC AGTTAACATC TGGAGGTGCC TCAGTCGTGT
480

GCTTCTTGAA CAACTTCTAC CCCAAAGACA TCAATGTCAA GTGGAAGATT GATGGCAGTG
540

AACGACAAAA TGGCGTCCTG AACAGTTGGA CTGATCAGGA CAGCAAAGAC AGCACCTACA
600

GCATGAGCAG CACCCTCACG TTGACCAAGG ACGAGTATGA ACGACATAAC AGCTATACCT
660

GTGAGGCCAC TCACAAGACA TCAACTTCAC CCATTGTCAA GAGCTTCAAC AGGAATGAGT
720

GTTAGAGACA AAGGTCCTGA GACGCCACCA CCAGCTCCCA GCTCCATCCT ATCTTCCCTT
780

```
CTAAGGTCTT GGAGGCTTCC CCACAAGCGC TTACCACTGT TGCGGTGCTC TAAACCTCCT      840

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(2) INFORMATION FOR SEQ ID NO: 5:

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          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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                 20                  25                  30

     Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser
             35                  40                  45

     Val Ser Tyr Met Asn Trp Tyr Gln Gln Lys Ser Gly Thr Ser Pro Lys
         50                  55                  60

     Arg Trp Ile Tyr Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala His
     65                  70                  75                  80

     Phe Arg Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Gly
                     85                  90                  95

     Met Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser
                 100                 105                 110

     Asn Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Asn Arg Ala
             115                 120                 125

     Asp Thr Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln Leu
         130                 135                 140

     Thr Ser Gly Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr Pro
     145                 150                 155                 160

     Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln Asn
                     165                 170                 175

     Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr Tyr
                 180                 185                 190

     Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg His
             195                 200                 205

     Asn Ser Tyr Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser Pro Ile
         210                 215                 220

     Val Lys Ser Phe Asn Arg Asn Glu Cys
     225                 230

(2) INFORMATION FOR SEQ ID NO: 6:
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1570 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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CTTTACTAGG TACACGATGC ACTGGGTAAA ACAGAGGCCT GGACAGGGTC TGGAATGGAT     240

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CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA GCCTGACATC     360

TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT GATCATTACT GCCTTGACTA     420

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CAAGGGTTAT TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG     600

TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA GCTCAGTGAC     660

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CAGCACCAAG GTGGACAAGA AAATTGAGCC CAGAGGGCCC ACAATCAAGC CCTGTCCTCC     780

ATGCAAATGC CCAGCACCTA ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA     840

GATCAAGGAT GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT     900

GAGCGAGGAT GACCCAGATG TCCAGATCAG CTGGTTTGTG AACAACGTGG AAGTACACAC     960

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CCCCATCCAG CACCAGGACT GGATGAGTCC CAAGGAGTTC AAATGCAAGG TCAACAACAA    1080

AGACCTCCCA GCGCCCATCG AGAGAACCAT CTCAAAACCC AAAGGGTCAG TAAGAGCTCC    1140

ACAGGTATAT GTCTTGCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC    1200

CTGCATGGTC ACAGACTTCA TGCCTGAAGA CATTTACGTG GAGTGGACCA ACAACGGGAA    1260

AACAGAGCTA AACTACAAGA ACACTGAACC AGTCCTGGAC TCTGATGGTT CTTACTTCAT    1320

GTACAGCAAG CTGAGAGTGG AAAAGAAGAA CTGGGTGGAA AGAAATAGCT ACTCCTGTTC    1380

AGTGGTCCAC GAGGGTCTGC ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTCCGGG    1440

TAAATGAGCT CAGCACCCAC AAAACTCTCA GGTCCAAAGA GACACCCACA CTCATCTCCA    1500

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AAAGGAATTC                                                           1570
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(2) INFORMATION FOR SEQ ID NO: 7:

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     (B) TYPE: amino acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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            20                  25                  30

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        35                  40                  45

Thr Arg Tyr Thr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
    50                  55                  60

Glu Trp Ile Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn
65                  70                  75                  80

Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser
                85                  90                  95

Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp
        115                 120                 125

Gly Gln Gly Thr Thr Leu Thr Val Ser Ser Ala Lys Thr Thr Ala Pro
    130                 135                 140

Ser Val Tyr Pro Leu Ala Pro Val Cys Gly Asp Thr Thr Gly Ser Ser
145                 150                 155                 160

Val Thr Leu Gly Cys Leu Val Lys Gly Tyr Phe Pro Glu Pro Val Thr
                165                 170                 175

Leu Thr Trp Asn Ser Gly Ser Leu Ser Ser Gly Val His Thr Phe Pro
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Ala Val Leu Gln Ser Asp Leu Tyr Thr Leu Ser Ser Ser Val Thr Val
        195                 200                 205

Thr Ser Ser Thr Trp Pro Ser Gln Ser Ile Thr Cys Asn Val Ala His
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Pro Ala Ser Ser Thr Lys Val Asp Lys Lys Ile Glu Pro Arg Gly Pro
225                 230                 235                 240

Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro Ala Pro Asn Leu Leu
                245                 250                 255

Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Ile Lys Asp Val Leu
            260                 265                 270

Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val Val Val Asp Val Ser
        275                 280                 285

Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe Val Asn Asn Val Glu
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    290                 295                 300

Val His Thr Ala Gln Thr Gln Thr His Arg Glu Asp Tyr Asn Ser Thr
305                 310                 315                 320

Leu Arg Val Val Ser Ala Leu Pro Ile Gln His Gln Asp Trp Met Ser
                325                 330                 335

Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys Asp Leu Pro Ala Pro
            340                 345                 350

Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser Val Arg Ala Pro Gln
        355                 360                 365

Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met Thr Lys Lys Gln Val
    370                 375                 380

Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro Glu Asp Ile Tyr Val
385                 390                 395                 400

Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn Tyr Lys Asn Thr Glu
                405                 410                 415

Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met Tyr Ser Lys Leu Arg
            420                 425                 430

Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser Tyr Ser Cys Ser Val
        435                 440                 445

Val His Glu Gly Leu His Asn His His Thr Thr Lys Ser Phe Ser Arg
    450                 455                 460

Thr Pro Gly Lys
465
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
1               5                   10                  15

Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Lys Ser Gly Thr Ser Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala His Phe Arg Gly Ser
    50                  55                  60

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Gly Met Glu Ala Glu
65                  70                  75                  80

Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95
```

Phe Gly Ser Gly Thr Lys Leu Glu Ile Asn Arg
100 105

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr
20 25 30

Leu Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Glu Ala Ser Asn Leu Gln Ala Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Gln Ser Leu Pro Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
100 105

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala
1 5 10 15

Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
20 25 30

Thr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Thr Asn Gln Lys Phe
50 55 60

Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser Thr Ala Tyr
65 70 75 80

```
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Phe Ile Phe Ser Ser Tyr
            20                  25                  30

Ala Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Ile Ile Trp Asp Asp Gly Ser Asp Gln His Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Asp Gly Gly His Gly Phe Cys Ser Ser Ala Ser Cys Phe Gly
            100                 105                 110

Pro Asp Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ser
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala
1               5                   10                  15

Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30
```

```
Thr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

```
          (A) LENGTH: 119 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein


     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

      Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
      1               5                   10                  15

      Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                  20                  25                  30

      Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
              35                  40                  45

      Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
          50                  55                  60

      Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Asn Thr Ala Phe
      65                  70                  75                  80

      Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                      85                  90                  95

      Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
                  100                 105                 110

      Thr Thr Leu Thr Val Ser Ser
              115

(2) INFORMATION FOR SEQ ID NO: 17:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 119 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein


     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

      Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
      1               5                   10                  15

      Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                  20                  25                  30

      Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
              35                  40                  45

      Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
          50                  55                  60

      Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Ala Phe
      65                  70                  75                  80

      Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                      85                  90                  95
```

```
Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45
```

```
Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Val
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
    50                  55                  60

Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
65                  70                  75                  80

Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala
                85                  90                  95

Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
            100                 105                 110

Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
    50                  55                  60

Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
65                  70                  75                  80

Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala
                85                  90                  95

Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
            100                 105                 110

Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid

```
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

     Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
     1               5                   10                  15

     Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Tyr Thr Phe Thr Arg Tyr
                 20                  25                  30

     Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
             35                  40                  45

     Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
         50                  55                  60

     Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
     65                  70                  75                  80

     Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala
                     85                  90                  95

     Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
                 100                 105                 110

     Thr Leu Thr Val Ser Ser
             115

(2) INFORMATION FOR SEQ ID NO: 24:

    (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 118 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

     Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
     1               5                   10                  15

     Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
                 20                  25                  30

     Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
             35                  40                  45

     Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
         50                  55                  60

     Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
     65                  70                  75                  80

     Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys Ala
                     85                  90                  95

     Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
                 100                 105                 110
```

```
Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
    50                  55                  60

Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe Leu
65                  70                  75                  80

Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys Ala
                85                  90                  95

Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
            100                 105                 110

Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Lys Val Lys
    50                  55                  60
```

```
Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Asn Thr Ala Phe Leu
65                  70                  75                  80

Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys Ala
                85                  90                  95

Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly Thr
            100                 105                 110

Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Phe Ile Phe Ser Ser Tyr
            20                  25                  30

Ala Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Ile Ile Trp Asp Asp Gly Ser Asp Gln His Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Asp Gly Gly His Gly Phe Cys Ser Ser Ala Ser Cys Phe Gly
            100                 105                 110

Pro Asp Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ser
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

```
Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
1               5                   10                  15
```

```
Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Lys Ser Gly Thr Ser Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala His Phe Arg Gly Ser
    50                  55                  60

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Gly Met Glu Ala Glu
65                  70                  75                  80

Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Ser Gly Thr Lys Leu Glu Ile Asn Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

```
Gln Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

```
Gln Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Glu Ala Ser Asn Leu Gln Ala Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Gln Ser Leu Pro Tyr
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

Claims

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

2. A CDR-grafted heavy chain according to Claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.

3. A CDR-grafted heavy chain according to Claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

4. A CDR-grafted heavy chain according to Claim 2 or 3, comprising donor residues at one, some or all of positions:
1 and 3,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.

6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to Claim 6 comprising donor residues at positions 46 and 47.

8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

9. A CDR-grafted light chain according to Claim 8 comprising donor residues at positions 46, 48, 58 and 71.

10. A CDR-grafted light chain according to Claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

11. A CDR-grafted light chain according to Claim 9 or 10, comprising donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form a potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 if different between donor and acceptor), and
any one or more of 10, 12, 40, 83, 103 and 105.

12. A CDR-grafted light chain according to any one of Claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of Claims 1-5 and at least one CDR-grafted light chain according to any one of Claims 6-12.

14. A CDR-grafted antibody molecule according to Claim 13, which is a site-specific antibody molecule.

15. A CDR-grafted antibody molecule according to Claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.

16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.

17. A DNA sequence which codes for a CDR-grafted heavy chain according to Claim 1 or a CDR-grafted light chain according to Claim 6 or Claim 8.

18. A cloning or expression vector containing a DNA sequence according to Claim 17.

19. A host cell transformed with a DNA sequence according to Claim 17.

20. A process for the production of a CDR-grafted antibody sequence according to Claim 17 in a transformed host cell.

21. A process for producing a CDR-grafted antibody product comprising:
    (a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to Claim 1;
  and/or
    (b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to Claim 6 or Claim 8;
    (c) transfecting a host cell with the or each vector;
  and
    (d) culturing the transfected cell line to produce the CDR-grafted antibody product.

22. A therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

23. A method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 to a human or animal subject.

```
  1  GAATTCCCAA AGACAAAatg gattttcaag tgcagatttt cagcttcctg
 51  ctaatcagtg cctcagtcat aatatccaga ggacaaattg ttctcaccca
101  gtctccagca atcatgtctg catctccagg ggagaaggtc accatgacct
151  gcagtgccag ctcaagtgta agttacatga actggtacca gcagaagtca
201  ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
251  agtccctgct cacttcaggg gcagtgggtc tgggacctct tactctctca
301  caatcagcgg catggaggct gaagatgctg ccacttatta ctgccagcag
351  tggagtagta acccattcac gttcggctcg gggacaaagt tggaaataaa
401  ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
451  agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
501  cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
551  tggcgtcctg aacagttgga ctgatcagga cagcaaagac agcacctaca
601  gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
651  agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
701  gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
751  CCAGCTCCCA GCTCCATCCT ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
801  CCACAAGCGC tTACCACTGT TGCGGTGCTC tAAACCTCCT CCCACCTCCT
851  TCTCCTCCTC CTCCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
901  AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA
```

Fig. 1(a)

```
  1  MDFQVQIFSF LLISASVIIS RGQIVLTQSP AIMSASPGEK VTMTCSASSS
 51  VSYMNWYQQK SGTSPKRWIY DTSKLASGVP AHFRGSGSGT SYSLTISGME
101  AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
151  ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
201  TLTKDEYERH NSYTCEATHK TSTSPIVKSF NRNEC*
```

Fig. 1(b)

```
   1 GAATTCCCCT CTCCACAGAC ACTGAAAACT CTGACTCAAC ATGGAAAGGC
  51 ACTGGATCTT TCTACTCCTG TTGTCAGTAA CTGCAGGTGT CCACTCCCAG
 101 GTCCAGCTGC AGCAGTCTGG GGCTGAACTG GCAAGACCTG GGGCCTCAGT
 151 GAAGATGTCC TGCAAGGCTT CTGGCTACAC CTTTACTAGG TACACGATGC
 201 ACTGGGTAAA ACAGAGGCCT GGACAGGGTC TGGAATGGAT TGGATACATT
 251 AATCCTAGCC GTGGTTATAC TAATTACAAT CAGAAGTTCA AGGACAAGGC
 301 CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA
 351 GCCTGACATC TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT
 401 GATCATTACT GCCTTGACTA CTGGGGCCAA GGCACCACTC TCACAGTCTC
 451 CTCAGCCAAA ACAACAGCCC CATCGGTCTA TCCACTGGCC CCTGTGTGTG
 501 GAGATACAAC TGGCTCCTCG GTGACTCTAG GATGCCTGGT CAAGGGTTAT
 551 TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG
 601 TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA
 651 GCTCAGTGAC TGTAACCTCG AGCACCTGGC CCAGCCAGTC CATCACCTGC
 701 AATGTGGCCC ACCCGGCAAG CAGCACCAAG GTGGACAAGA AAATTGAGCC
 751 CAGAGGGCCC ACAATCAAGC CCTGTCCTCC ATGCAAATGC CCAGCACCTA
 801 ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA GATCAAGGAT
 851 GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT
 901 GAGCGAGGAT GACCCAGATG TCCAGATCAG CTGGTTTGTG AACAACGTGG
 951 AAGTACACAC AGCTCAGACA CAAACCCATA GAGAGGATTA CAACAGTACT
1001 CTCCGGGTGG TCAGTGCCCT CCCCATCCAG CACCAGGACT GGATGAGTGG
1051 CAAGGAGTTC AAATGCAAGG TCAACAACAA AGACCTCCCA GCGCCCATCG
1101 AGAGAACCAT CTCAAAACCC AAAGGGTCAG TAAGAGCTCC ACAGGTATAT
1151 GTCTTGCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC
1201 CTGCATGGTC ACAGACTTCA TGCCTGAAGA CATTTACGTG GAGTGGACCA
1251 ACAACGGGAA AACAGAGCTA AACTACAAGA ACACTGAACC AGTCCTGGAC
1301 TCTGATGGTT CTTACTTCAT GTACAGCAAG CTGAGAGTGG AAAAGAAGAA
1351 CTGGGTGGAA AGAAATAGCT ACTCCTGTTC AGTGGTCCAC GAGGGTCTGC
1401 ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTCCGGG TAAATGAGCT
1451 CAGCACCCAC AAAACTCTCA GGTCCAAAGA GACACCCACA CTCATCTCCA
1501 TGCTTCCCTT GTATAAATAA AGCACCCAGC AATGCCTGGG ACCATGTAAA
1551  AAAAAAAAAA AAAGGAATTC
```

Fig. 2(a)

```
OKT 3 HEAVY CHAIN PROTEIN SEQUENCE DEDUCED FROM DNA SEQUENCE

  1  MERHWIFLLL LSVTAGVHSQ VQLQQSGAEL ARPGASVKMS CKASGYTFTR
 51  YTMHWVKQRP GQGLEWIGYI NPSRGYTNYN QKFKDKATLT TDKSSSTAYM
101  QLSSLTSEDS AVYYCARYYD DHYCLDYWGQ GTTLTVSSAK TTAPSVYPLA
151  PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGSLSSGVHT FPAVLQSDLY
201  TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC
251  PAPNLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWFV
301  NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMSGKEF KCKVNNKDLP
351  APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV
401  EWTNNGKTEL NYKNTEPVLD SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH
451   EGLHNHHTTK SFSRTPGK*
```

Fig. 2(b)

```
           1                    23                 42
           NN      N              N        N    N
RES TYPE   SBspSPESssBSbSsSssPSPSPsPSsse*s*p*Pi^ISsSe
Okt3vl     QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
REI        DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK
           ? ?
             CDR1    (LOOP)            *******
             CDR1    (KABAT)         ***********


                       56                          85
           N  NN
RES TYPE   *IsiPpIeesesssSBEsePsPSBSSEsPspsPsseesSPePb
Okt3vl     SPKRWIYDTSKLASGVPAEFRGSGSGTSYSLTISGMEAEDAAT
REI        APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT
           ?  ??                      ?  ?
                 *******  CDR2 (LOOP/KABAT)

                         102   108
RES TYPE   PiPIPies**iPIIsPPS?SPSS
Okt3vl     YYCQQWSSNPFTFGSGTKLEINR
REIvl      YYCQQYQSLPYTFGQGTKLQITR
                         ?    ?
               ******           CDR3 (LOOP)
             **********         CRD3 (KABAT)
```

Fig. 3

```
          NN N                 23 26    32 35 N39  43
RES TYPE  SESPs^SBssS^sSSsSpSpSPsPSEbSBssBePiPIpiesss
Okt3h     QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ
KOL       QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
               ?                ??
                                     ******    CDR1 (LOOP)
                                         ***** CDR1 (KABAT)


                   52a     60 65     N N  N     82abc    89
RES TYPE IIeIppp^ssssssss^ps^pSSsbSpseSsSseSp^pSpsSBssS^ePb
Okt3vh   GLEWIGYINPSRGYTNTNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAV
KOL      GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLRPEDTGV
             ??                     ? ?  ?  ?            ?
                ************        CDR2  (LOOP)
                ******************** CDR2  (KABAT)


           92 N                  107    113
RES TYPE  PiPIEissssiiisssbibi*EIPIP*spSBSS
Okt3vh    YYCARYYDDHY.......CLDYWGQGTTLTVSS
KOL       YFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS
               ***************** CRD3 (KABAT/LOOP)
```

Fig. 4

OKT 3 HEAVY CHAIN CDR GRAFTS

1. gh341 and derivatives

```
         1                            26        35   39   43
Okt3vh   QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ
gH341    QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK   JA178
gH341A   QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA185

gH341E   QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA198
gH341*   QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA207
gH341*   QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA209
gH341D   QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA197
gH341*   QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA199
gH341C   QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA184

gH341*   QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA203
gH341*   QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA205
gH341B   QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK   JA183
gH341*   QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA204
gH341*   QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA206
gH341*   QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA208
KOL      QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
```

Fig. 5(i)

```
         44    50             65               83
Okt3vh   GLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLT
gH341    GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR JA178
gH341A   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA185

gH341E   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA198
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTAFLQMDSLR JA207
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTAFLQMDSLR JA209
gH341D   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTLFLQMDSLR JA197
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTLFLQMDSLR JA199
gH341C   GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR JA184

gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA207
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA205
gH341B   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA183
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA204
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA206
gH341*   GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTAFLQMDSLR JA208
KOL      GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLR
```

Fig. 5(ii)

```
          84         95              102        113
Okt3vh    SEDSAVYYCARYYDDHY.......CLDYWGQGTTLTVSS
gH341     PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA178
gH341A    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS    JA185

gH341E    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA198
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA207
gH341D    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA197
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA209
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA199
gH341C    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA184

gH341*    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS    JA203
gH341*    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS    JA205
gH341B    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS    JA183
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA204
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA206
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS    JA208
KOL       PEDTGVYFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS
```

Fig. 5(iii)

OKT3 LIGHT CHAIN CDR GRAFTING

1. gL221 and derivatives

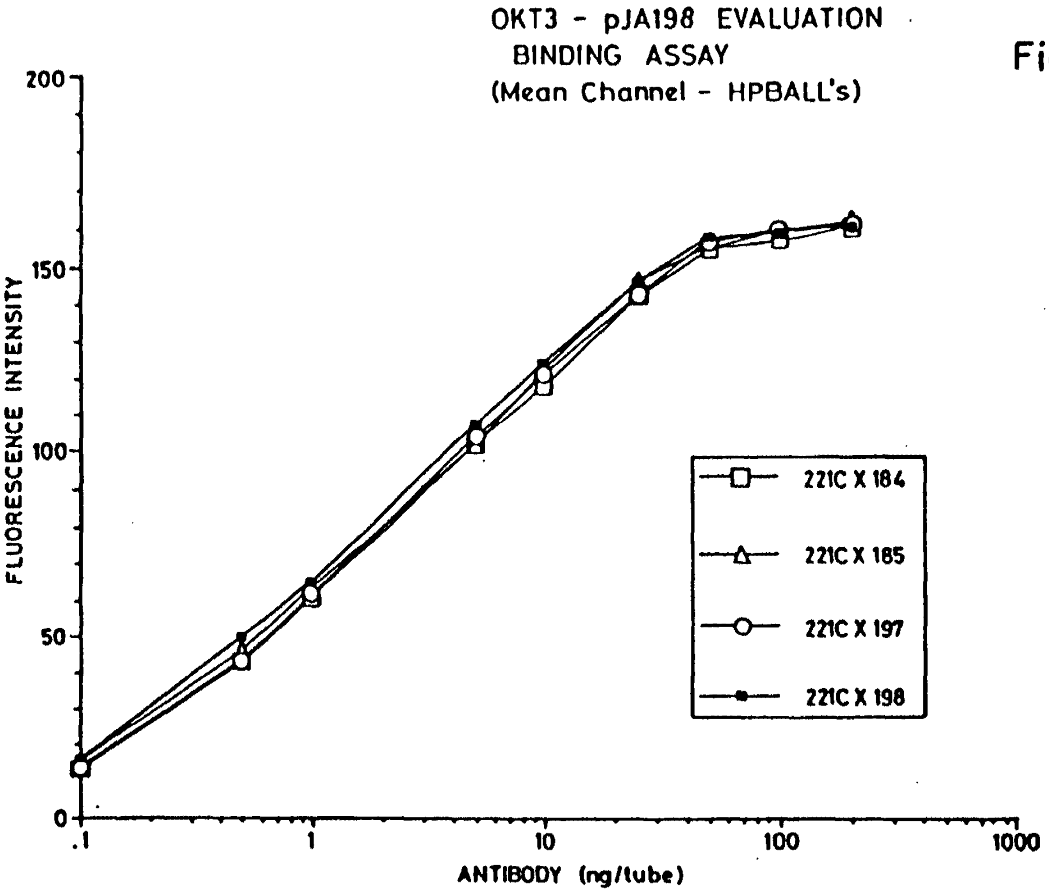
```
           1                      24        34      42
Okt3vl     QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
gL221      DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221A     QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221B     QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221C     DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
REI        DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK

           43     50    56                           85
Okt3vl     SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
gL221      APKLLIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221A     APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221B     APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221C     APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
REI        APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT

           86   91   96          108
Okt3vl     YYCQQWSSNPFTFGSGTKLEINR
gL221      YYCQQWSSNPFTFGQGTKLQITR
gL221A     YYCQQWSSNPFTFGQGTKLQITR
gL221B     YYCQQWSSNPFTFGQGTKLQITR
gL221C     YYCQQWSSNPFTFGQGTKLQITR
REI        YYCQQYQSLPYTFGQGTKLQITR
```

CDR'S ARE UNDERLINED

FRAMEWORK RESIDUES INCLUDED IN THE GENE ARE DOUBLE UNDERLINED

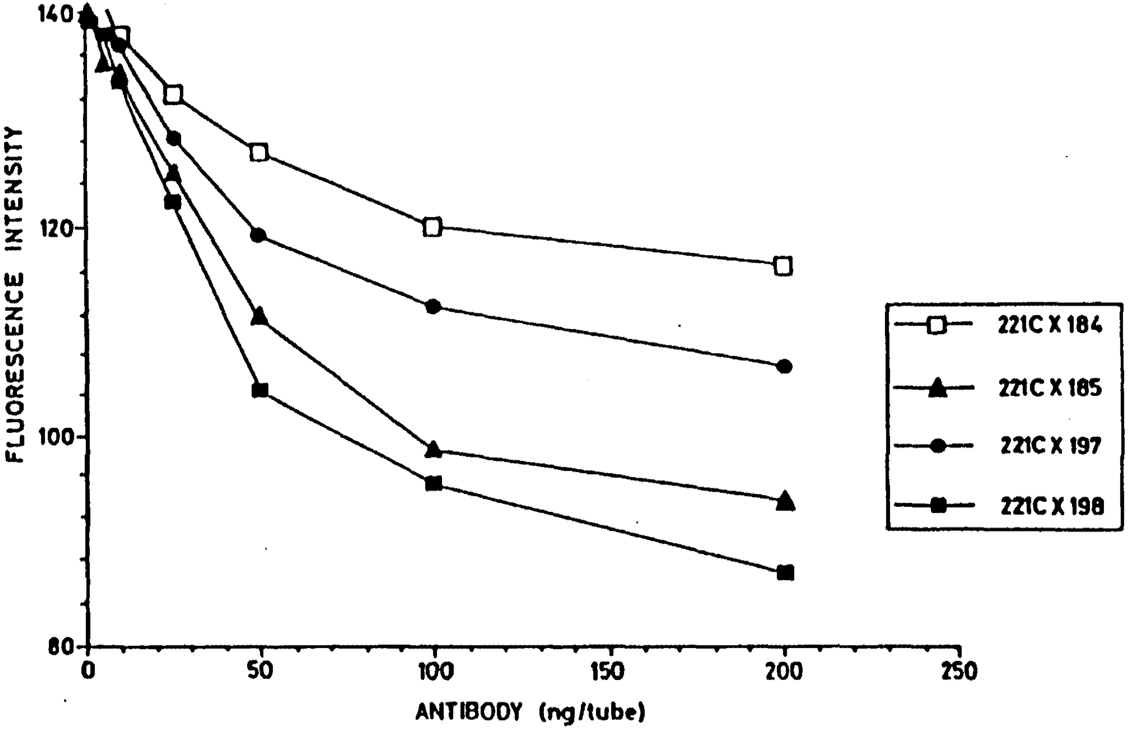
Fig. 6

Fig. 7


OKT3 - pJA198 EVALUATION
BINDING ASSAY
(Mean Channel - HPBALL's)
FLUORESCENCE INTENSITY
200
150
100
50
0
.1
1
10
100
1000
ANTIBODY (ng/tube)
221C X 184
221C X 185
221C X 197
221C X 198

OKT3 - pJA198 EVALUATION
BLOCKING ASSAY
(Mean Channel -HPBALL's)

Fig. 8



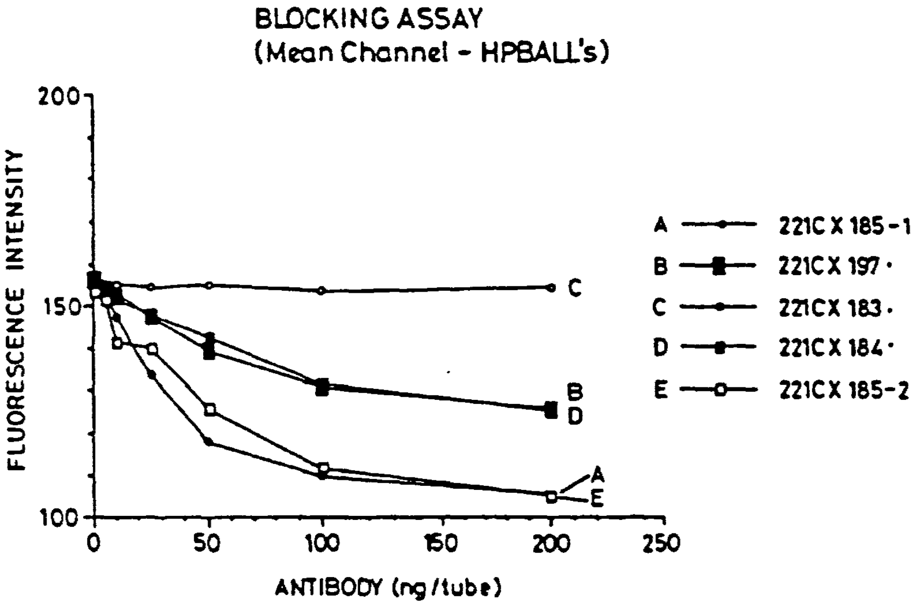

BLOCKING ASSAY
(Mean Channel - HPBALL's)
FLUORESCENCE INTENSITY
200
150
100
0
50
100
150
200
250
ANTIBODY (ng/tube)
A 221C X 185-1
B 221CX 197 ·
C 221CX 183 ·
D 221CX 184 ·
E 221CX 185-2
C
B
D
A
E

Fig. 9

Fig.10

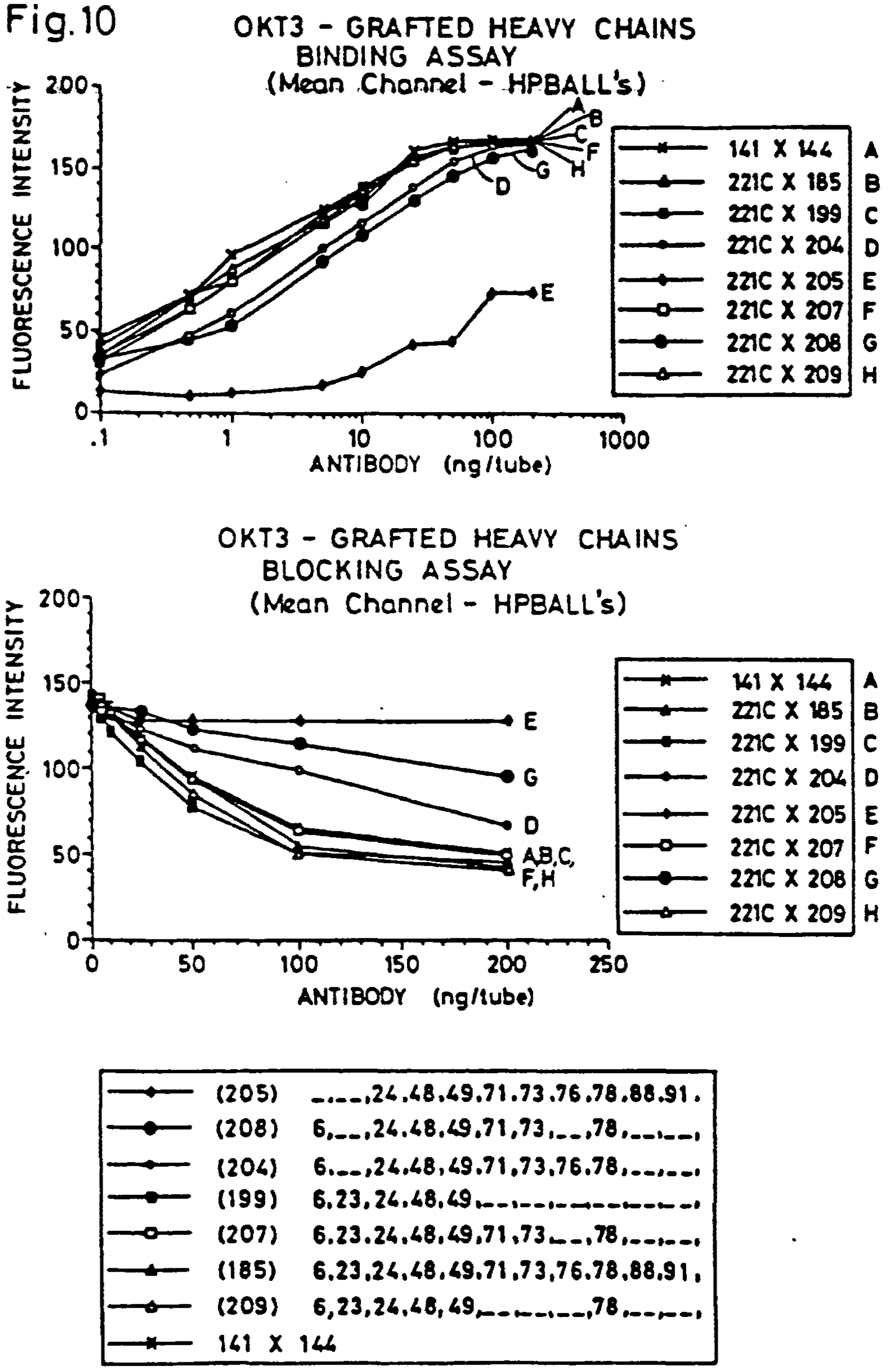
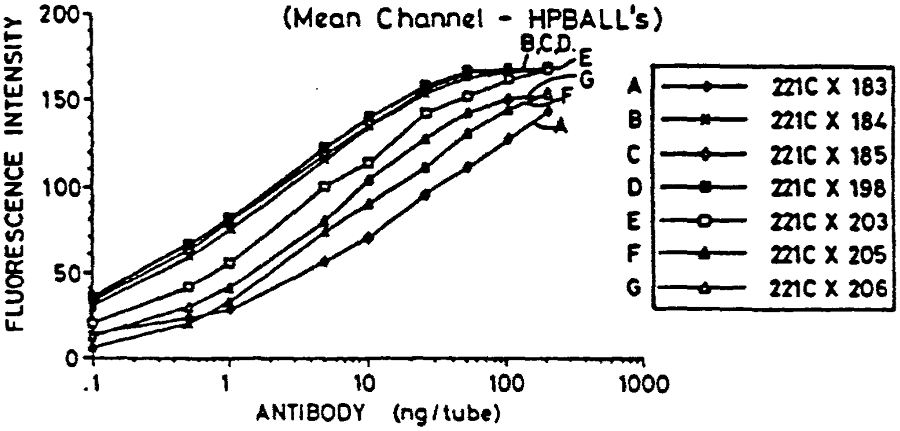
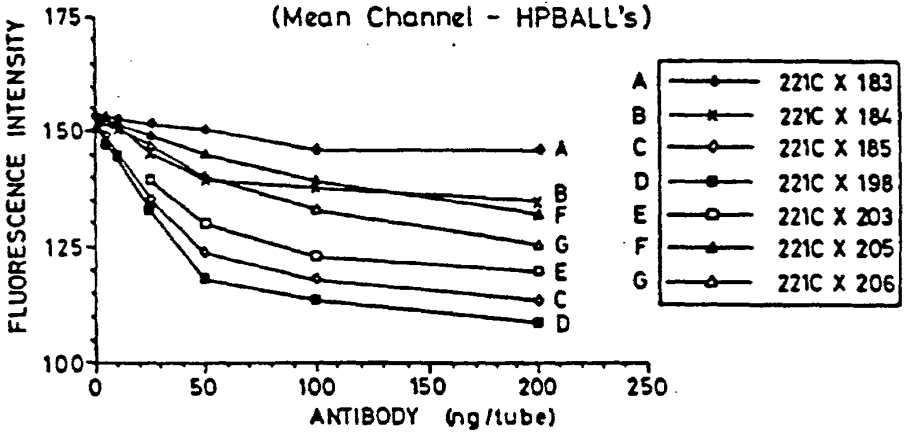

OKT3 - GRAFTED HEAVY CHAINS
BINDING ASSAY
(Mean Channel - HPBALL's)
FLUORESCENCE INTENSITY
200
150
100
50
0
.1
1
10
100
1000
ANTIBODY (ng/tube)
A
B
C
D
E
F
G
H
141 X 144 A
221C X 185 B
221C X 199 C
221C X 204 D
221C X 205 E
221C X 207 F
221C X 208 G
221C X 209 H
OKT3 - GRAFTED HEAVY CHAINS
BLOCKING ASSAY
(Mean Channel - HPBALL's)
FLUORESCENCE INTENSITY
200
150
100
50
0
0
50
100
150
200
250
ANTIBODY (ng/tube)
E
G
D
A,B,C,
F,H
141 X 144 A
221C X 185 B
221C X 199 C
221C X 204 D
221C X 205 E
221C X 207 F
221C X 208 G
221C X 209 H
(205) _,__,24,48,49,71,73,76,78,88,91,
(208) 6,__,24,48,49,71,73,__,78,__,__,
(204) 6,__,24,48,49,71,73,76,78,__,__,
(199) 6,23,24,48,49,__,__,__,__,__,__,
(207) 6,23,24,48,49,71,73,__,78,__,__,
(185) 6,23,24,48,49,71,73,76,78,88,91,
(209) 6,23,24,48,49,__,__,__,78,__,__,
141 X 144

Fig. 11

OKT3 - GRAFTED HEAVY CHAINS
BINDING ASSAY
(Mean Channel - HPBALL's)



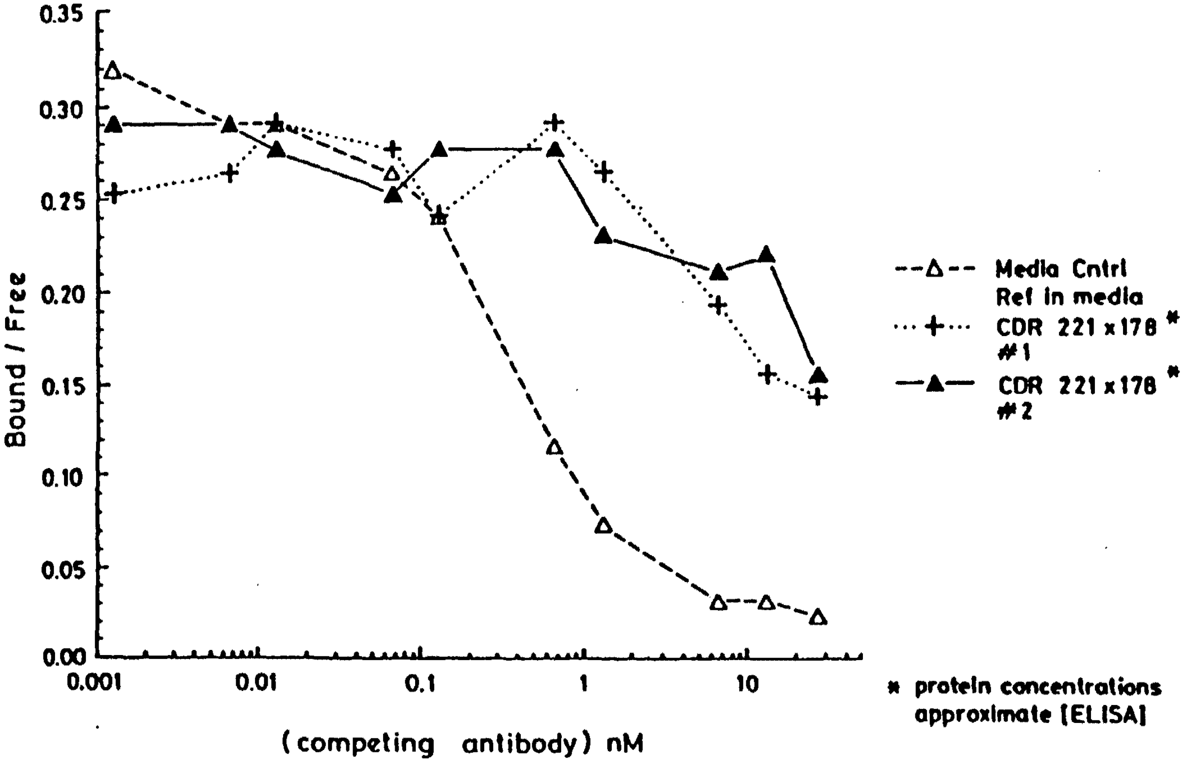
OKT3 GRAFTED HEAVY CHAINS
BLOCKING ASSAY
(Mean Channel - HPBALL's)



◆	(183)	_,_,_,48,49,71,73,76,78,88,91,
▲	(205)	_,_,24,48,49,71,73,76,78,88,91,
✱	(184)	6,23,24,_,_,_,_,_,_,_,_,
△	(206)	_,_,24,48,49,71,73,76,78,_,_,
□	(203)	6,_,24,48,49,71,73,76,78,88,91,
◇	(185)	6,23,24,48,49,71,73,76,78,88,91,
■	(198)	6,23,24,48,49,71,73,76,78,_,_,

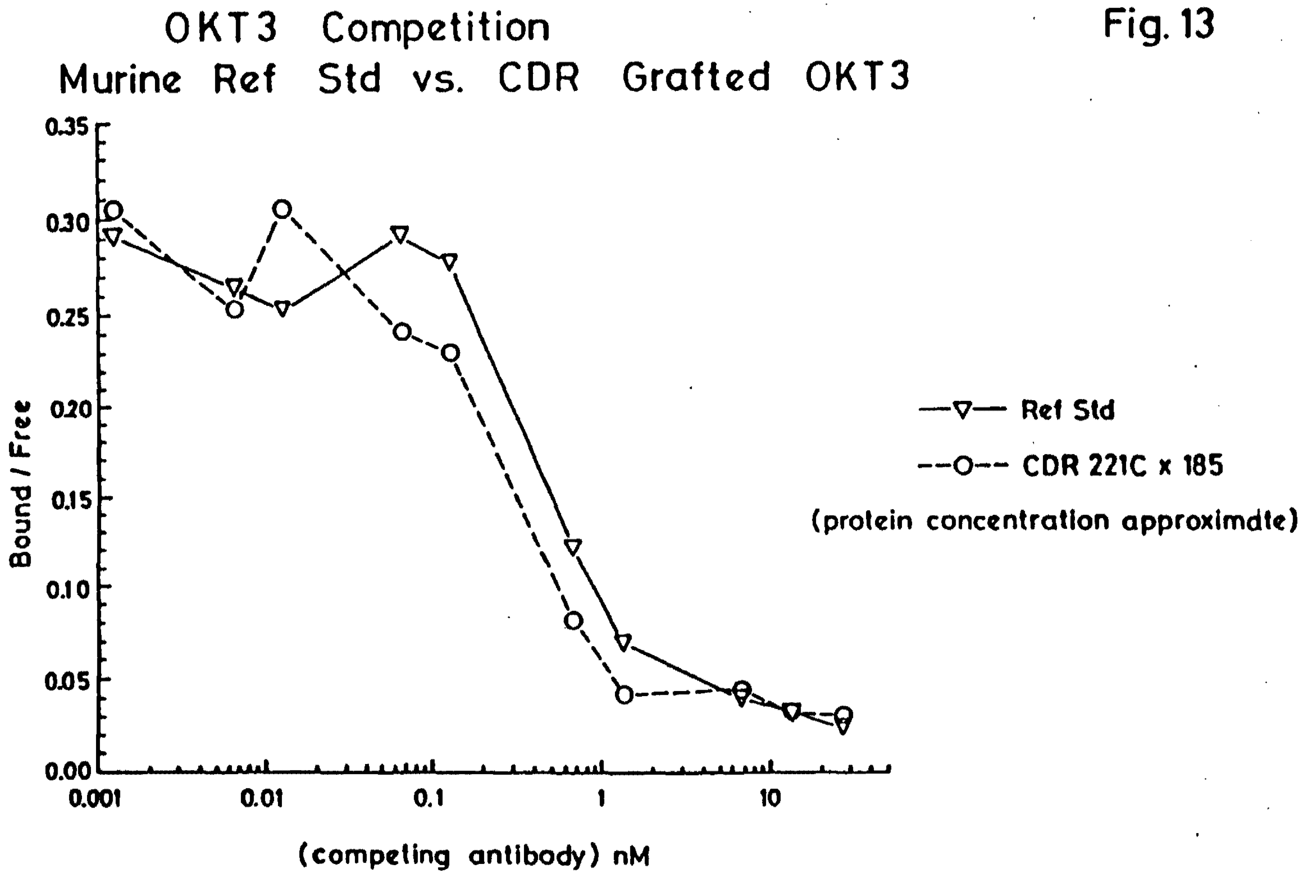
Fig. 12

OKT3 Competition
Murine Ref Std vs. CDR Grafted OKT3



* protein concentrations approximate [ELISA]

Fig. 13


OKT3 Competition
Murine Ref Std vs. CDR Grafted OKT3
Bound / Free
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.001
0.01
0.1
1
10
(competing antibody) nM
—▽— Ref Std
--○-- CDR 221C x 185
(protein concentration approximdte)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number
EP 94 10 4042

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
D,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA vol. 86, no. 24 , 15 December 1989 , WASHINGTON, DC, USA pages 10029 - 10033 C. QUEEN ET AL. 'A humanized antibody that binds to the interleukin 2 receptor.' * the whole document * ---	1-23	C12N15/13 C07K15/28 A61K39/395 G01N33/577
P,X	EP-A-0 403 156 (GENZYME CORPORATION & BEHRINGWERKE) * the whole document * -----	1-23	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)

C12N
C07K
A61K
G01N

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
THE HAGUE	8 June 1994	Nooij, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

EP 94 10 4042

-C-

Remark: Although claim 23
is directed to a method of
treatment of (diagnostic method
practised on) the human/animal body
(Art. 52(4) EPC) the search has been
carried out and based on the
alleged effects of the compound/
composition

PCT
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :		(11) International Publication Number:	WO 89/01783
A61K 39/395, C12N 15/00 C12P 21/00	A2	(43) International Publication Date:	9 March 1989 (09.03.89)

(21) International Application Number: PCT/GB88/00731

(22) International Filing Date: 5 September 1988 (05.09.88)

(31) Priority Application Number: 8720833

(32) Priority Date: 4 September 1987 (04.09.87)

(33) Priority Country: GB

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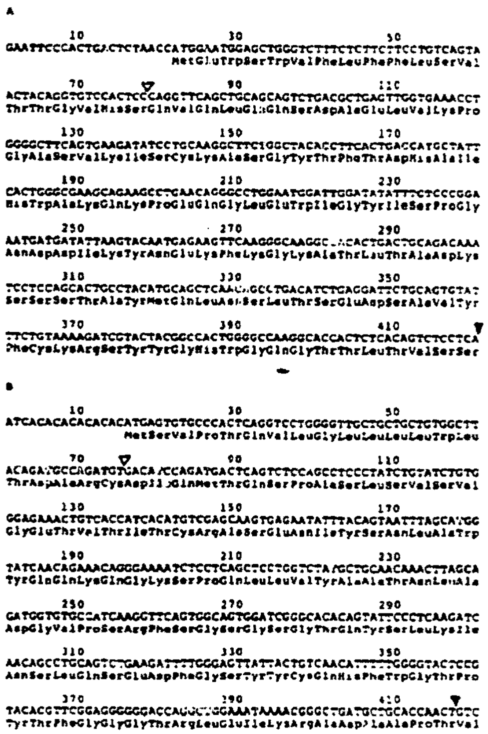
(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, RO, SE (European patent), SU, US.

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RECOMBINANT ANTIBODY AND METHOD

(57) Abstract

The present invention provides a humanised antibody molecule (HAM) having specificity for the TAG-72 antigen and having an antigen binding site wherein at least the complementarity determining regions (CDRs) of the variable domain are derived from the mouse monoclonal antibody B72.3 (B72.3 MAb) and the remaining immunoglobulin-derived parts of the HAM are derived from a human immunoglobulin and a process for its production.



Carter et al. P0709P1
SN: 08/146,206
Filed November 17, 1993

RECOMBINANT ANTIBODY AND METHOD

The present invention relates to a humanised antibody molecule (HAM) having specificity for an antigen present on certain malignant cells and to a process for its production using recombinant DNA technology.

In the present application, the term "recombinant antibody molecule" (RAM) is used to describe an antibody produced by any process involving the use of recombinant DNA technology, including any analogues of natural immunoglobulins or their fragments. The term "humanised antibody molecule" (HAM) is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, the remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site may comprise either complete variable domains fused onto constant domains or only the complementarity determining regions grafted onto appropriate framework regions in the variable domains. The abbreviation "MAb" is used to indicate a monoclonal antibody.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, $(Fab')_2$ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site at the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, have been hindered by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential of immunoglobulins as therapeutic agents was the discovery of monoclonal antibodies of defined specificity (1). However, most MAbs are produced by fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness.

There have therefore been made proposals for making non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanizing" MAbs. These techniques generally involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Some early methods for carrying out such a procedure are described in EP-A-0 171 496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), EP-A-0 194 276 (Celltech Limited) and WO-A-8 702 671 (Int. Gen. Eng. Inc.). The Celltech application discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. It also shows the production of an antibody molecule comprising the variable domains of a mouse MAb, the CH1 and CL domains of a human immunoglobulin, and a non-immunoglobulin-derived protein in place of the Fc portion of the human immunoglobulin.

In an alternative approach, described in EP-A-87302620.7 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin using site directed mutagenesis using long oligonucleotides .

The earliest work on humanizing MAbs has been carried out based on MAbs recognising synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAb recognising lysozyme and a rat MAb recognising an antigen on human T cells respectively were humanized are shown by Verhoeyen et al. (2) and Reichmann et al. (3) .

It has been widely suggested that immunoglobulins, and in particular MAbs, could potentially be very useful in the diagnosis and treatment of cancer (4,5). There has therefore been much activity in trying to produce immunoglobulins or MAbs directed against tumour-specific antigens. So far, over one hundred MAbs directed against a variety of human carcinomas have been used in various aspects of tumour diagnosis or treatment (6).

There have been a number of papers published concerning the production of chimeric monoclonal antibodies recognising cell surface antigens. For instance, Sahagan et al. (7) disclose a genetically engineered murine/human chimeric antibody which retains specificity for a human tumour-associated antigen. Also Nishimura et al. (8) disclose a recombinant murine/human chimeric monoclonal antibody specific for common acute lymphocytic leukemia antigen.

According to the present invention, there is provided a humanised antibody molecule (HAM) having specificity for the TAG-72 antigen and having an antigen binding site wherein at least the complementarity determining regions (CDRs) of the variable domain are derived from the mouse monoclonal antibody B72.3 (B72.3 MAb) and the remaining immunoglobulin-derived parts of the HAM are derived from a human immunoglobulin.

The variable domains of the HAM may comprise either the entire variable domains of the B72.3 MAb or may comprise the framework regions of a human variable domain having grafted thereon the CDRs of the B72.3 MAb.

The B72.3 MAb is a mouse MAb of the type IgG1-Kappa raised against a membrane-enriched extract of a human liver metastatis of a breast carcinoma (9). The B72.3 MAb has been extensively studied in a number of laboratories. It has been shown to recognise a tumour-associated glycoprotein TAG-72, a mucin-like molecule with a molecular weight of approximately 10^6 (10). Immunohistochemical studies have demonstrated that the B72.3 MAb recognises approximately 90% of colorectal carcinomas, 85% of breast carcinomas and 95% of ovarian carcinomas. However, it shows no significant cross-reactivity with a wide spectrum of normal human tissues (11 to 14).

It has surprisingly been found that humanizing the B72.3 MAb does not adversely affect its binding activity, and this produces a HAM which is extremely useful in both therapy and diagnosis of certain carcinomas.

Preferably, the HAM of the present invention will be produced by recombinant DNA technology.

The HAM of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as the Fab or $(Fab')_2$ fragment; a light chain or heavy chain dimer; or any other molecule with the same specificity as the B72.3 antibody.

Alternatively, the HAM of the present invention may have attached to it an effector or reporter molecule. For instance, the HAM may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce a HAM in which the Fc fragment or CH3 domain of a complete antibody molecule has been replaced by an enzyme or toxin molecule.

The remainder of the HAM may be derived from any suitable human immunoglobulin. However, it need not comprise only protein sequences from the human immunoglobulin. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a polypeptide effector or reporter molecule.

According to a second aspect of the present invention, there is provided a process for producing the HAM of the first aspect of the invention, which process comprises:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain wherein at least the CDRs of the variable domain are derived from the B72.3 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain wherein at least the CDRs of the variable domain are derived from the B72.3 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the HAM.

The cell line may be transfected with two vectors, the first vector containing an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical except in so far as the coding sequences and selectable markers are concerned so as to ensure as far as possible that each polypeptide chain is equally expressed.

Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially genomic DNA. Most preferably, the heavy or light chain encoding sequence comprises a fusion of cDNA and genomic DNA.

The host cell used to express the HAM of the present invention is preferably a eukaryotic cell, most preferably a mammalian cell, such as a CHO cell or a myeloid cell. It has been found, surprisingly, that the use of cDNA/genomic DNA fusions for the heavy or light chain coding sequences leads to enhanced production of the HAM of the present invention in non-myeloid mammalian cells. Thus, an important aspect of the invention is the use of such fusions in non-myeloid mammalian cells in order to express the HAM.

The present invention also includes cloning and expression vectors and transfected cell lines used in the process of the invention, therapeutic and diagnostic compositions containing the HAM of the invention and uses of such compositions in therapy and diagnosis.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known _per se_ and form no part of the invention. Such methods are shown, for instance, in references 15 and 16.

The present invention is now described, by way of example only, with reference to the accompanying drawings, in which:-

Figure 1 shows the DNA sequences encoding the unprocessed variable regions of the B72.3 MAb obtained by sequencing the cDNA clones pBH41 and pBL52. Panel A shows the sequence coding for the VH region and the predicted amino acid sequence. Panel B shows the sequence coding for the VL region and the first 21 residues of the CL region, together with the predicted amino acid sequence. The points of fusion with the human C regions are indicated with arrows.

The putative sites of cleavage of the signal peptide are indicated by open triangles. The numbers refer to the nucleotides in the original cDNA clones;

Figure 2 is a schematic diagram of the construction by site directed mutagenesis, restriction and ligation of the chimaeric heavy chain gene;

Figure 3 is a schematic diagram of the construction by partial restriction and ligation of the chimaeric light chain gene;

(In Figures 2 and 3, coding sequences are shown as boxes, dark for the variable regions and light for the constant regions. Restriction enzymes are abbreviated as follows: EcoRI=E; BglII=B; HindIII=H; MboII=M; HpaI=Hp; and ScaI=Sc. Dotted lines indicate the continuation of the sequence into vector or constant region DNA.)

Figure 4 is a schematic diagram of the hCMV expression vector and the four alternative cDNA or gene constructs inserted into the EcoRI site. The chimaeric heavy chain gene was inserted using a BamHI-EcoRI oligonucleotide adapter. Coding sequences are represented by boxes, dark for the variable regions and light for the constant regions. The direction of transcription is indicated with an arrow;

Figure 5 shows an ELISA analysis of COS-cell transfectant supernatants. The level of antigen-binding capacity in the supernatant of COS-cell transfectants was analysed as described later. Dilution curves were plotted out against the optical density of the colour change. Different antibodies were used to recognise mouse or human epitopes, and consequently the antigen-binding levels for each curve are not strictly comparable. Each curve represents a co-transfection, as follows: △ mouse heavy chain, mouse light chain; ▲ mouse heavy chain, chimaeric light chain; □ chimaeric heavy chain, mouse light chain; ■ chimaeric heavy chain, chimaeric light chain;

Figure 6 shows SDS-PAGE analysis in a reducing gel of immunoprecipitations from the supernatants of transfected COS-cells. The DNA used for the transfection was as follows: Lane 1, mouse light chain alone; Lane 2, mouse light chain, mouse heavy chain; Lane 3, mouse light chain, chimaeric heavy chain; Lane 4, chimaeric light chain alone; Lane 5, chimaeric light chain, mouse heavy chain; and Lane 6, chimaeric light chain, chimaeric heavy chain. The antibodies used for the immunoprecipitations were: Lanes 1-3, rabbit anti-mouse $F(ab')_2$; Lanes 4-6, rabbit anti-human $F(ab')_2$;

Figure 7 shows SDS-PAGE analysis of immunoprecipitations from the supernatants of transfected COS-cells, under non-reducing (lanes 1-3), and reducing (lanes 4-6) conditions. The DNA used for transfection was as follows: lanes 1 and 4, chimaeric light chain clone; lanes 2 and 5, chimaeric light chain, mouse heavy chain; lanes 3 and 6, chimaeric light chain, chimaeric heavy chain. The antibody used for the immunoprecipitation in each case was rabbit anti-human $F(ab')_2$;

Figure 8 shows SDS-PAGE analysis on a reducing gel of immunoprecipitations from the supernatants of transfected COS-cells, grown and labelled in the absence (lanes 1 and 3), and the presence (lanes 2 and 4) of tunicamycin. The DNA used for the transfections was as follows: lanes 1 and 2, chimaeric light chain clone; and lanes 3 and 4, chimaeric light chain and chimaeric heavy chain. The antibody used for immunoprecipitation in each case was rabbit anti-human $F(ab')_2$;

Figure 9 shows reducing and non-reducing SDS-PAGE gels of chimeric B72.3 produced by CHO cells;

Figure 10 shows a two dimensional SDS-PAGE gel of chimeric B72.3 produced by CHO cells;

Figure 11 shows a time course study of tumour labelling using B72.3 antibodies;

Figure 12 shows the tissue/tumour ratio of the B72.3 antibodies; and

Figure 13 shows the construction of plasmid TR002

EXAMPLE 1

Molecular cloning and sequencing of the B72.3 heavy and light chain cDNAs.

Polyadenylated RNA was isolated from the B72.3 hybridoma cell line using the guanidinium isothiocyanate/caesium chloride method (15). Double stranded cDNA was synthesised (17) and a cDNA library was constructed in bacteriophage λgt 10 vector using EcoRI linkers (18). Two screening probes were synthesised, complementary to mouse immunoglobulin heavy and light chain constant regions. The heavy chain probe was a 19 mer complementary to residues 115-133 in the CH1 domain of the mouse $\gamma 1$ sequence (19). The light chain probe was a 20 mer complementary to residues 4658-4677 of the genomic mouse CK sequence (20). The probes were radio-labelled at the 5' terminus with [γ ^{32}P] ATP using T4 polynucleotide kinase (Amersham International) and used to screen the cDNA library.

Clones which contained the complete leader, variable and constant regions of both the heavy and light chains were isolated. The EcoRI cDNA inserts were subcloned into M13mp8 vectors for sequencing (21), generating a heavy chain clone, designated pBH41, and a light chain clone, designated pBL52. Nucleotide sequence analysis was carried out according to the chain termination procedure (22).

The 980 base pair EcoRI insert in pBL52 was fully sequenced (22). The EcoRI insert in pBH41 was shown to comprise approximately 1700 base pairs by agarose gel electrophoresis. The variable domain and the 5' region of the CH1 domain were sequenced, as was the 3' end of the clone to confirm the presence of the correct mouse γ1 termination sequences. The DNA and predicted amino acid sequences for the unprocessed variable regions of pBH41 and pBL52 are shown in Figure 1. Examination of the derived amino acid sequence revealed considerable homology with other characterised immunoglobulin genes, and enabled the extent of the leader, variable and constant domains to be accurately determined. In addition, MAb B72.3 was confirmed to be an IgG1 K antibody, as previously reported (9).

Construction of the Chimaeric Mouse-Human Heavy Chain Clone

A genomic clone containing sequences coding for the human Cγ4 region was isolated as a HindIII fragment from the cosmid COS Ig8 (23) and then cloned via pAT153 into M13tg130 as an EcoRI-BamHI fragment to form pJA78. Following DNA sequence analysis, an 18 mer oligonucleotide was synthesised and site specific mutagenesis was performed to convert a C residue to an A residue, thereby generating a novel HindIII site at the start of the CH1 exon, to yield pJA91.

Site directed mutagenesis was performed (24) using EcoRI- and BglI-cut M13mp18 to generate a gapped duplex with the relevant phage template. DNA was transformed into E. coli HB2154 and resultant transformants were propagated on E. coli HB2151 (Anglian Biotechnology Ltd) as described in the protocols provided. All mutations were sequenced using the chain termination procedure (22). All sequenced fragments were subsequently recloned into other vectors in order to exclude the possibility of secondary mutations which may have occurred during the mutagenesis procedure.

The VH domain from the B72.3 heavy chain cDNA, cloned in M13mp9 as pBH41, was isolated as an EcoRI-BglI fragment and introduced into the EcoRI-HindIII sites of pJA91 in conjunction with a 32 base pair BglI-HindIII adaptor to yield pJA93. The product was therefore a chimaeric immunoglobulin heavy chain gene containing a variable region derived from a mouse cDNA clone fused to a sequence, comprising the CH1, H, CH2 and CH3 domains separated by introns, derived from a human genomic clone. The accuracy of the variable/constant region junction was confirmed by nucleotide sequence analysis. A schematic diagram of details of the construction is given in Figure 2. The γ4 constant region was selected as it possesses a limited repertoire of effector functions, but does bind to staphylococcal protein A, a potentially useful reagent for purification.

Construction of the Chimaeric Mouse-Human Light Chain Gene

The mouse light chain cDNA clone, pBL52, contains a cutting site for MboII 18 base pairs downstream from the junction of the variable and constant domains. Due to sequence homology between the mouse and human CK genes, an identical cutting site exists in the latter gene (25) and use of this site provides a method of fusing the mouse variable and human constant domains. Partial digestion of the EcoRI fragment containing the mouse cDNA clone with MboII generated a 416 base pair EcoRI-MboII fragment with a single residue overhang. A genomic clone, comprising an M13-derived vector containing the human C-kappa gene on a PstI-HindIII fragment was digested with FokI. A 395 base pair fragment containing the majority of C-kappa was cloned into pAT153 using EcoRI linkers to form pNW200. Digestion of a 945 base pair ScaI-HindIII fragment from pNW200 with MboII generated a 374 base pair MboII-HindIII fragment, which could anneal with and be ligated to the 416 base pair EcoRI-MboII fragment described above. The two fragments were ligated into a pSP64 vector linearised with EcoRI and HindIII, and used to transform competent E. coli HB101. The variable/constant region junction was sequenced in order to confirm the correct fusion. The construction is outlined schematically in Figure 3.

Construction of Expression Vectors for Transient Expression in COS Cells

The heavy and light chain chimaeric genes, as well as the mouse heavy and light chain cDNA clones, were inserted separately into the unique EcoRI site of plasmid pEE6 (27). The light chain encoding plasmid was designated EE6.cL.neo. For the chimaeric heavy chain, this was accomplished by using an oligonucleotide adapter to change the 3' BamHI site to an EcoRI site to give an EcoRI fragment for cloning. The heavy chain encoding plasmid was designated EE6.cH.gpt. This plasmid contains the strong promoter/enhancer and transcriptional control element from the human cytomegalovirus (hCMV) inserted into a unique HindIII site upstream of the EcoRI site. In addition, an SV40 origin of replication site is provided by the SV40 early promoter which drives a selectable marker gene, either a neomycin-resistance gene (neo) for light chain genes or a guanine phosphoribosyl transferase gene (gpt) for heavy chain genes, inserted into a unique BamHI site. The plasmid also contains an ampicillin-resistance gene allowing selection and propagation in bacterial hosts. The structures of the expression vector and immunoglobulin gene inserts are shown schematically in Figure 4.

Transfections and ELISA Analysis of Antibody Production

The four expression constructs described above were used singly or in heavy/light chain gene pairs to transfect COS-1 cells (26). The cells were left to incubate in DNA-DEAE dextran solution for six hours, then shocked for two minutes with 10% DMSO in HEPES-buffered saline. The cells were washed and incubated in medium containing 10% foetal calf serum for 72 hours.

Following incubation at 37°C for 72 hours the cell supernatants and lysates were analysed by ELISA for heavy and light chain production and binding of antigen.

The medium (500 µl per 10^5 cells) was removed for ELISA analysis. Cell lysates were prepared by lysis of 10^5 cells in 500 µl 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01M sodium phosphate pH 7.5, 0.1M sodium chloride and 0.001M EDTA. Lysates and conditioned medium were centrifuged for 5 minutes in an Eppendorf centrifuge to remove nuclei and cell debris, and stored at 4°C before analysis.

Microtitre plates were coated with 0.25 µg per well of sheep or goat antibody reactive against either human or mouse specific epitopes on the heavy or light chains. Supernatants or lysates from transfected COS cells were diluted 1:2 or 1:4 respectively in sample conjugate buffer containing 0.1M Tris-HCl pH 7.0, 0.1M sodium chloride, 0.02% Tween 20 and 0.2% casein. 100 µl of each diluted sample were added to each well and incubated for 1 hour at room temperature with gentle agitation. Following washing six times with wash buffer (phosphate buffered saline containing 0.2% Tween 20, pH 7.2), 100 µl of 1:5000 dilution of standard horseradish peroxidase - conjugated antibody reactive against either human or mouse specific epitopes were added per well. The plates were incubated for 1 hour at room temperature, and then washed six times with wash buffer. 100 µl of substrate buffer containing 0.1 mg/ml tetramethylbenzidine (TMB), 0.1M sodium citrate, pH 6.0 and 0.005% H_2O_2 were added to each well to generate a colour change. The reaction was terminated after 2-3 minutes by adjusting the solution to pH 1.0 with 1.5M sulphuric acid. The optical density was determined at 450nm for each well by measurement in a Dynatech laboratories MR600 microplate reader. Standard curves were generated using known concentrations of the appropriate human or mouse immunoglobulins.

Antigen binding assays were performed in an analogous manner. Microtitre plates were coated with 0.25 µg per well of purified TAG-72 antigen (6) obtained from human patients or from tumour xenografts implanted in nude mice (both gifts of J. Schlom, NCI). Following washing six times in wash buffer, samples from COS-cell transfections were added as previously, and the same subsequent procedures carried out, using goat anti-mouse or human F(ab')$_2$ linked to HRP as the second antibody.

A number of assay systems using different capture antibodies were developed and cross-correlated to investigate the potential products of each transfection. In all cases, mouse light chain and chimaeric light chain were detected in the supernatants and lysates of appropriately transfected cells. However heavy chains were only detected in the supernatants when co-transfected with light chain. A low level of heavy chain was detected in the cell lysate in each case, supporting a suggestion of inhibition of heavy chain secretion in the absence of light chain.

Assembly assays, which detect the presence of associated polypeptide chains, demonstrated the formation of multimers containing at least one heavy and one light chain when both genes were co-transfected. Mouse genes and chimaeric genes appeared equally capable of assembly and formation of hybrid molecules.

Antigen binding analysis (see above) demonstrated that the mouse heavy and light chain co-transfections generated an antibody molecule capable of recognising antigen. Replacement of the mouse gene for either chain with the appropriate chimaeric gene led to the production of a hybrid molecule with antigen binding specificity in the ELISA assays. Finally, transfection of the COS cells with both the chimaeric heavy and light chain genes generated a complete chimaeric antibody molecule with antigen binding specificity. The ELISA data from one experiment are presented in Figure 5. These experiments demonstrate that "humanisation" of the antibody molecule does not have a significant effect on its antigen recognition capability.

Immunoprecipitation of Antibody Molecules from Biosynthetically Labelled COS-Cell Transfectants

Preliminary experiments suggested that there was little expression from the transfected DNA in the initial 24 hours. Accordingly following transfection, COS cells were allowed to recover for 24 hours in DMEM containing 10% foetal calf serum. The medium was then replaced with methionine-free DMEM, to which $[^{35}S]$ methionine (NEN) had been added at 200 µCi/ml. The cells were metabolically labelled for 48 hours, and conditioned media and cell lysates prepared as previously.

Analysis by reducing SDS-PAGE of aliquots of COS cell supernatant demonstrated the appearance of labelled immunoglobulin protein without further purification, while use of Protein A-Sepharose was shown to selectively precipitate whole antibody, but not light chain alone, from the COS-cell supernatant.

Further analysis of the assembly and secretion of antibody molecules was performed by immunoprecipitation using anti-human $F(ab')_2$ and anti-human C-kappa anti-sera bound to Protein A-Sepharose. Affinity-purified rabbit antibodies against human IgG $F(ab')_2$ and human K chain were used for immunoprecipitations, following coupling to Protein A - Sepharose. Both cytoplasmic and secreted antibodies were analysed on an SDS-10% PAGE system under reducing and non-reducing conditions. The gel was treated with an autoradiography enhancer, dried and exposed to Fuji RX film. The results are shown in Figure 6.

Both antisera immunoprecipitated proteins with an apparent molecular weight of 55K and 28K, which coincided with the positions of the Coomassie-stained immunoprecipitating heavy and light immunoglobulin chains respectively. The use of the latter antisera demonstrated that light chain is found associated with heavy chain in the COS-cell supernatants. A comparison of immunoprecipitations analysed by reducing and non-reducing SDS-PAGE (See Figure 7) suggests that the heavy and light chains are assembled as the correct tetrameric molecule. In addition there is evidence for the secretion of free light chain dimers and partially assembled heavy and light chain multimers.

Due to the presence of secondary structure caused by disulphide bonds, the mobility of the immunoglobulin chains is different in the two systems. In order to analyse the presence and potential role of glycosylation, COS cells were treated with tunicamycin, at the same time as the radioactive label was added. To inhibit N-linked glycosylation, COS cells were grown in medium containing 10 μg/ml tunicamycin diluted from a stock solution. To ensure that the pool of lipid-linked oligosaccharide was depleted, the cells were maintained in the tunicamycin-containing medium for 2 hours prior to addition of radioactive label.

Following immunoprecipitation the protein products were analysed by SDS-PAGE, as shown in Figure 8. It is clear from the decrease in apparent molecular weight, from 55K to 52K, that the chimaeric heavy chain, but not the light chain, undergoes N-linked glycosylation. When the glycosylation is inhibited, the protein is still secreted, although the level of expression appears to be decreased.

These results demonstrate that each of the immunoglobulin genes is correctly transcribed and translated. The two mouse genes and the chimaeric light chain are cDNA-like, while the chimaeric heavy chain gene possesses characteristics of both cDNA and genomic DNA. Both types of construct appear to be expressed at a similar level and with similar fidelity. It is clear therefore that transcript splicing occurs where necessary, but it is not an obligatory requirement for correct expression of immunoglobulin genes in COS-cells.

The expressed heavy and light chains associate in the correct manner, presumably limited by the availability of free polypeptide chain. Mouse and human polypeptide sequences appear interchangeable in the association of heavy and light chains. The product is an assembled tetrameric antibody molecule, which is expressed at a high level, glycosylated and secreted into the culture medium.

Development of Stable Cell Lines in CHO Cells

Stable Light Chain Producing Cell Line

Chinese hamster ovary (CHO-K1) cells were grown in attached culture in Dulbecco's Modified Eagles Medium (DMEM) containing 10% foetal calf serum (FCS), non-essential amino acids (NEAA) and glutamine (2mM). A confluent culture was trypsinised, the cells washed once in phosphate buffered saline (PBS) and resuspended at 10^7 cells/ml.

DNA from plasmid EE6.cL.neo, comprising the chimaeric light chain gene expressed from the HCMV promoter, was digested with SalI to generate linear molecules and then ethanol precipitated. The precipitate was resuspended in PBS and 40 μg of DNA was added to 10^7 CHO-K1 cells in buffer at 4°C. The DNA was introduced into the cells by electroporation, in which the cell suspension and DNA were treated with two pulses of 2000 volts. After electroporation the cells were returned to 4°C for 10 min prior to plating out at a density of 5×10^5 cells per 90mm Petri dish in DMEM growth medium containing supplements.

Following incubation at 37°C overnight, selection for introduced DNA was applied by adding G418 to a final concentration of 1mg/ml. Resistant colonies were observed after 10-14 days incubation in selective medium.

Resistant colonies were picked from the transformation plates using filter paper squares soaked in trypsin solution and transferred into individual wells of 24 well tissue culture plates. The culture medium from the wells was assayed for the presence of chimaeric light chain using an ELISA assay and cell lines secreting light chain were identified. Lines producing light chain at levels between 100ng/ml and 16 μg/ml were identified. Those producing the highest amounts were cloned out by the limiting dilution method. One such clone, cL18, was used for subsequent studies.

Stable Chimaeric Antibody Producing Cell Line

DNA from plasmid EE6.cH.gpt (also designated as JA96), comprising the chimaeric heavy chain gene expressed from the HCMV promoter, was digested with SalI to generate linear molecules and then ethanol prepicitated. CHO-cL18 cells producing chimaeric light chain were prepared for electroporation as described above, and the JA96 DNA (40 μg per 10^7 cells) was introduced by electroporation. Following overnight incubation in non-selective medium, selection for resistance to mycophenolic acid was applied. Selective medium comprised DMEM, 10% FCS, NEAA, glutamine, xanthine, hypoxanthine, thymine and mycophenolic acid (10 μg/ml). Resistant colonies were detected after 10-14 days and these were picked into 24 well plates as described above. Antibody producing cell lines were identified using an antigen binding assay based on the antigen TAG-72 recognised by the antibody B72.3. Cell lines producing antibody at levels ranging from 0.1-40 μg/ml were isolated. Two of these cell lines, F6 and F11, were used in further studies.

Purification of Chimaeric Antibody

Chimaeric B72.3 was purified from CHO cell culture supernatant by affinity chromatography using Protein-A Sepharose and ion-exchange chromatography. Cell culture supernatant was adjusted to pH 8.8 with sodium glycinate (0.2M) and applied to a column of Protein-A Sepharose pre-equilibrated with glycine/glycinate buffer at pH 8.8 (50mM). After sample loading, the column was washed with equilibration buffer and the antibody eluted with a gradient of decreasing pH made up of disodium hydrogen phosphate (0.2M) and citric acid (0.1M). Fractions containing chimaeric antibody were pooled, dialyzed into 50 mM phosphate buffer pH 8.0 and then applied to a column of DEAE-Sepharose pre-equilibrated with 50mM phosphate buffer pH 8.0. The column was washed with equilibration buffer and elution of antibody achieved with a linear gradient of sodium chloride from 0.0 to 0.2M. Purified antibody was then dialyzed into an appropriate buffer for the intended use, e.g. PBS for animal studies, and concentrated by ultrafiltration. Typical yields of chimaeric antibody were 20 mg per litre of starting supernatant.

Purity and assembly of the chimaeric antibody was tested by SDS-polyacrylamide gel electrophoresis (PAGE), both reducing and non-reducing (Fig 9), and by HPLC gel filtration.

N-terminal amino acid sequencing of the antibody revealed a single sequence encoding light chain, which corresponded exactly to the expected amino acid sequence deduced from the DNA sequence. The heavy chain was N-terminally blocked and not amenable to amino acid sequencing. Antigen binding activity was demonstrated in an ELISA format assay.

Chimaeric B72.3 made in COS or CHO cells contains a proportion of material (10-20%) which fails to form an inter-heavy chain disulphide bridge but otherwise assembles correctly into 150kD molecules containing two heavy and two light chains. This material is present in antibody when secreted from the cell and co-purifies with antibody in which the inter-heavy chain disulphide bridges have formed. This appears to be a common property of human IgG4 molecules and occurs with all molecules of this type analysed to date, including a mouse-human IgG4 chimaeric anti-NP antibody and two different IgG4 myeloma proteins.

On non-reducing SDS-polyacrylamide gel electrophoresis of chimaeric B72.3, two bands are seen, one at the expected size of 150 kD and one of about 80 kD (Fig. 9) Both of these bands contain intact heavy and light chains as shown by non-reducing/reducing two dimensional SDS-PAGE (Fig. 10). Reducing SDS-PAGE shows only intact heavy and light chain (Fig. 9). Native (non SDS) electrophoresis and HPLC gel filtration show only one species corresponding to 150 kD material. Thus the about 80 kD band seen on non-reducing SDS-PAGE represents materials with a molecular weight of 150 kD in solution. The two halves of the molecule are only separated when other inter-heavy chain interactions are disrupted, e.g. when run on non-reducing SDS-PAGE

Efficacy Studies

The chimaeric B72.3 antibody is capable of being used to advantage in a number of circumstances. For example, after suitable labelling by radioisotopes or other detection procedure, the antibody can be demonstrated to locate and bind *in vivo* to solid tumours where some or all of the tumour cells express the specific antigen TAG-72. The experiment described below is one example of the ability of the chimaeric antibody to locate human tumour cells bearing the specific antigen, in this case in a nude mouse model system.

Chimaeric B72.3 and mouse B72.3 antibodies were radioactively labelled with ^{125}I by the Chloramine

T method to an approximate specific activity of 5 µCi/µg. Groups of 4 female nude mice bearing subcutaneous LS174T xenografts on the flank were injected intravenously with 100 µCi of either Chimaeric B72.3 or mouse B72.3 in 0.1ml PBS. Groups of animals were sacrificed at intervals for the collection of tissue samples, which were weighed, dissolved in 7M potassium hydroxide and counted in an LKB model 1270 "Rackbeta" counter. Tissue uptake was calculated as the mean percentage of injected dose per gram of tissue from a group of four animals.

Fig. 11 shows a time course study of the mouse and chimaeric antibodies and demonstrates clearance of the antibodies from the blood pool and uptake at the tumour site. The chimaeric antibody appears to clear somewhat faster from the blood pool but locates to the tumour adequately with approximately the same profile as the mouse antibody. This sample data suggests that the novel engineered antibody is functional *in vivo*.

Fig. 12 shows the tumour to tissue ratio at 24, 48 and 168 h. It can be seen that tumour/tissue ratios increase with time and that in this model system the chimaeric antibody has a superior tumour/tissue ratio compared to the mouse antibody.

EXAMPLE 2

Chimaeric B72.3 - Other IgG Isotypes

Construction of Chimaeric Antibody Genes

Assembly of Chimaeric Antibody Genes

Genomic DNA sequences containing the human IgG1, 2 and 3 genes were isolated from larger DNA

inserts in phage λ and were introduced into phage M13 via pJA103 which contains the human IgG4 gene with a HindIII site at the 5' end of the CH1 exon and a BamHI 3' to the CH3 exon. The M13 vector is M13tg130 which has two amber mutations in essential genes and is therefore suitable for high efficiency site-directed mutagenesis experiments using the procedures described earlier. A HindIII site was introduced at the 5' end of the CH1 exon in each isotype gene to give pRB11 (IgG1), pRB14 (IgG2) and pRB16 (IgG3). SalI and BglII sites were also introduced into pRB11 towards the 3' end of the CH1 exon and towards the 3' end of the intron following the CH1 exon respectively. The isotypes were then reisolated as HindIII-BamHI fragments and sub-cloned into pAT153 to give RB18 (IgG1), RB26 (IgG2), and RB20 (IgG3). The B72.3 VH DNA sequence was isolated and was ligated to the linking oligonucleotide which was used earlier to make the IgG4 chimaeric heavy chain gene so as to give an EcoRI-HindIII VH fragment. This fragment was ligated to the human IgG1 HindIII-BamHI containing fragment of RB18 and cloned in pAT153 to give pRB22.
To construct the chimaeric B72.3 VH, the VH fragment described above was ligated to the HindIII-BamHI fragment of pRB26 and recloned in pAT153 to give pRB27.

To construct the chimaeirc B72.3 VH/IgG3 gene, the VH fragment described above was ligated to the HindIII-BamHI fragment of pRB20 and recloned in pAT153 to give pRB23.

Assembly of Genes in Expression Vectors

The chimaeric genes were isolated as EcoRI-BamHI fragments from pRB22, 27 and 23 described above and cloned between the EcoRI and BclI sites of JA96, the B72.3 IgG4 chimaeric heavy chain expression vector, thus replacing the IgG4 chimaeric gene. The resultant chimaeric expression plasmids were named RB24 (IgG1 chimaera), RB28 (IgG2 chimaera) and RB25 (IgG3 chimaera)

Demonstration of production, assembly and activity were performed as in Example 1.

EXAMPLE 3

Chimaeric B72.3 IgG4 F(ab')$_2$

Construction of F(ab') Heavy Chain Gene

Assembly of F(ab') Gene

pJA79 is an M12tg130 vector which contains the human IgG4 heavy chain gene modified so that the sequence from the first nucleotide after the last codon of the hinge exon to the last nucleotide of the CH3 domain inclusive has been removed by oligonucleotide directed site specific deletion. The hinge and 3' untranslated region and part of the M13 sequence can be isolated as a 1.1 kbp BglII fragment. This fragment can be used to replace the analogous fragment in the full length B72.3/IgG4 chimaeric heavy chain gene clone pJA93 to give plasmid JA94 which therefore contains a chimaeric gene potentially capable of being expressed to produce a B72.3 IgG4 chimaeric F(ab') heavy chain protein.

Assembly of Gene in Expression Vector

Plasmid pJA94 described above was used to recover the F(ab') gene as an EcoRI-BamHI 1475 bp fragment. This fragment was cloned into the unique EcoRI site of the pEE6 expression vector using a BamHI to EcoRI oligonucleotide adapter to give pJA97.

Test of Genes in Cos Cells

The chimaeric F(ab') gene in pJA97 was expressed in COS cells in conjunction with a suitable construct capable of expressing of chimaeric light chain polypeptide as described above. PAGE analysis of the expression products and subsequent inspection of the DNA sequence of the CH1-hinge intron suggested that splicing out of the intron was not occurring correctly leading to the production of an aberrant heavy chain polypeptide.

Reconstruction of IgG4 F(ab') Heavy Chain Gene

Assembly of F(ab') Gene

pJA94 described above was derived from pJA93 which in turn was derived from pJA91. This clone was initially an M13tg130 based vector, i.e. an amber phage capable of being used in the efficient gap-heteroduplex mutagenesis procedure described earlier. In order to repeat the mutagenesis procedure at high yield, the chimaeric F(ab') heavy chain gene was isolated as an EcoRI fragment and recloned into M13tg130 to give pJA100. By oligonucleotide directed site specific mutagenesis, a SalI site was introduced towards the 3' end of the

CH1 exon to give pJA108. The introduced SalI site in the CH1 domain codes for the fifth and fourth from last amino acids of the CH1 domain. To reconstruct the hinge into the end of the CH1 domain, four oligonucleotides were made which together are able to code for the last five amino acids of the CH1 domain, the hinge sequence, two in-frame stop codons and an EcoRI site.

The oligonucleotides were assembled and cloned into M13 and mp11 between the SalI and EcoRI sites in the polylinker, sequenced, reisolated and ligated to the gene containing the EcoRI-SalI 700bp fragment from pJA108 to reconstruct the chimaeric B72.3 F(ab') heavy chain gene.

The reconstructed CH1/hinge sequence should be:

CH1 hinge

Asp Lys Arg Val Glu Ser Lys Tyr Gly Pro Pro Cys Pro
Ser Cys Pro Stop

The oligonucleotide used to form this CH1/hinge were

1. 5TCGACAAGAGAGTTGAGTCCAAATATGGG
2. 3'GTTCTCTCAACTCAGGTTTATACCCGGGGG

3. 5'CCCCCGTGCCCATCATGCCCATGATG
4. 3'CACGGGTAGTACGGGTACTACTTAA

In the vector, oligonucleotides 1 and 3 produced the sense strand and oligonucleotides 2 and 4 produced the anti-sense strand.

Assembly of Gene in Expression Vector

The chimaeric B72.3 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcoRI vector fragment of pJA96 to give pJA114.

Test of Genes in COS Cells

The genes were tested in COS cells as described above. On non-reducing SDS-PAGE the material appeared to be produced as F(ab') material only. Reducing SDS-PAGE showed the presence of light chain and truncated heavy chain equivalent to that expected from the F(ab') gene.

Development of Stable Cell Lines in CHO Cells

The expression plasmid pJA114, comprising the B72.3 chimaeric F(ab') heavy chain gene fragment expressed from the HCMV promoter, was introduced by electroporation into the CHO cell line CL18 described above. The procedure was similar to that described for introduction of the full length chimaeric heavy chain except that the SalI digestion was omitted and the DNA was introduced as closed circular DNA. Cell lines resistant to mycophenolic acid and expressing function F(ab') antibody were identified by screening culture supernatants in an antigen binding ELISA format assay as described earlier. Cell lines expressing between 0.1-6 μg/ml F(ab') were isolated. One cell line, FB9 was used for further studies.

Purification of Chimaeric $F(ab')_2$ Antibody

Chimaeric F(ab') was purified from CHO cell supernatant using immunopurification. An immunopurification reagent was prepared by linking NH3/41, an antibody was specificity for human Kappa chain sequence, to cyanogen bromide activated Sepharose by standard methodology. This material was packed into a column and equilibrated with PBS. CHO cell culture supernatant containing chimaeric F(ab') was applied to the column and the column was washed with PBS. Elution of chimaeric F(ab') was then achieved using 4.5M guanidine hydrochloride. Fractions containing chimaeric F(ab') were then dialyzed extensively into PBS and concentrated by ultrafiltration.

Purity and assembly of F(ab') was tested by SDS-PAGE (both reducing and nonreducing and by HPLC gel filtration. Antigen binding activity was demonstrated using an ELISA format assay. Approximately 10% of the material can be found as $F(ab')_2$ which forms without further treatment.

EXAMPLE 4

Chimaeric B72.3 IgG1 $F(ab')_2$

Construction of F(ab') Heavy Chain Gene

Example 2 discloses the vector RB22 which contains the B72.3/human IgG1 chimaeric cloned gene in pAT153. Vectors JA96 and JA108 are mentioned above. The plasmid TR002 containing hinge modified gene was constructed as shown in Figure 13. The chimaeric F(ab') region containing the B72.3 VH/IgG1 was isolated as a 0.7 kbp fragment from RB22 by treating the DNA with SalI, removing the 5' phosphate from the SalI site with calf intestinal phosphatase (CIP) and recutting the DNA with EcoRI.

The IgG1 hinge was assembled by kinase labelling 500pm of top and bottom strand oligonucleotide and annealing the oligonucleotides by heating to 70°C and cooling to room temperature in the kinase buffer. The hinge fragments were ligated to the 0.7 kbp fragment from JA108 prepared as above, and the CIP'ed 5' ends were kinased.

Assembly of Gene in Expression Vector

The chimaeric B72.3 IgG1 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcorI/CIP treated vector fragment of JA96 to give TR002. Expression of TR002 in suitable cells with an expression vector capable of producing a useful light chain, for example chimaeric or humanised B72.3 will produce material which should assemble to give F(ab') and which will on suitable post translational modification *in vivo* or *in vitro* give $F(ab')_2$.

Thus, it has been demonstrated that it is possible to produce a HAM having specificity derived from a mouse MAb but having human constant regions, and which may have an important note to play in cancer diagnosis and therapy.

It will be appreciated that the present invention has been described above by way of illustration only, and that variations or modifications of detail can be made without departing from the scope of the invention.

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CLAIMS

1. A humanised antibody molecule (HAM) having specificity for the TAG-72 antigen and having an antigen binding site wherein at least the complementarity determining regions (CDRs) of the variable domains are derived from the mouse monoclonal antibody B72.3 (B72.3 MAb) and the remaining immunoglobulin-derived parts of the HAM are derived from a human immunoglobulin.

2. The HAM of claim 1, wherein the entire variable domains are derived from the B72.3 MAb.

3. The HAM of claim 1 or claim 2, when produced by recombinant DNA technology.

4. The HAM of any one claims 1 to 3, which comprises a complete antibody molecule, an Fab fragment or an $(Fab')_2$, fragment.

5. The HAM of any one of claims 1 to 4, wherein an effector or reporter molecule is attached thereto.

6. The HAM of claim 5, wherein the effector or reporter molecule is a protein molecule which is coexpressed as a fusion protein with one of the chains of the HAM.

7. A process for producing the HAM of any one of claims 1 to 6, which process comprises

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain wherein at least the

CDRs of the variable domain are derived from the B72.3 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain wherein at least the CDRs of the variable domain are derived from the B72.3 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the HAM.

8. The process of claim 7, wherein the heavy and light chain encoding sequences are present on the same vector.

9. The process of claim 7, wherein the heavy and light chain encoding sequences are present on separate vectors.

10. The process of any one of claims 7 to 9, wherein the DNA coding sequences comprise fusions of cDNA and genomic DNA.

11. The process of claim 10, wherein the host cell is a non-myeloid mammalian cell.

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FIG. 1

A

```
         10                             30                             50
GAATTCCCACTGACTCTAACCATGGAATGGAGCTGGGTCTTTCTCTTCTTCCTGTCAGTA
                        MetGluTrpSerTrpValPheLeuPhePheLeuSerVal

         70         ▽                   90                            110
AC[illegible]CCACTCCCAGGTTCAGCTGCAGCAGTCTGACGCTGAGTTGGTGAAACCT
Thr[illegible]GlyValHisSerGlnValGlnLeuGlnGlnSerAspAlaGluLeuValLysPro

        130                            150                            170
GGGGCTTCAGTGAAGATATCCTGCAAGGCTTCTGGCTACACCTTCACTGACCATGCTATT
GlyAlaSerValLysIleSerCysLysAlaSerGlyTyrThrPheThrAspHisAlaIle

        190                            210                            230
CACTGGGCGAAGCAGAAGCCTGAACAGGGCCTGGAATGGATTGGATATATTTCTCCCGGA
HisTrpAlaLysGlnLysProGluGlnGlyLeuGluTrpIleGlyTyrIleSerProGly

        250                            270                            290
AATGATGATATTAAGTACAATGAGAAGTTCAAGGGCAAGGCCACACTGACTGCAGACAAA
AsnAspAspIleLysTyrAsnGluLysPheLysGlyLysAlaThrLeuThrAlaAspLys

        310                            330                            350
TCCTCCAGCACTGCCTACATGCAGCTCAACAGCCTGACATCTGAGGATTCTGCAGTGTAT
SerSerSerThrAlaTyrMetGlnLeuAsnSerLeuThrSerGluAspSerAlaValTyr

        370                            390                            410       ▼
TTCTGTAAAAGATCGTACTACGGCCACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
PheCysLysArgSerTyrTyrGlyHisTrpGlyGlnGlyThrThrLeuThrValSerSer
```

B

```
         10                             30                             50
ATCACACACACACACATGAGTGTGCCCACTCAGGTCCTGGGGTTGCTGCTGCTGTGGCTT
                  MetSerValProThrGlnValLeuGlyLeuLeuLeuLeuTrpLeu

         70    ▽                        90                            110
ACAGATGCCAGATGTGACATCCAGATGACTCAGTCTCCAGCCTCCCTATCTGTATCTGTG
ThrAspAlaArgCysAspIleGlnMetThrGlnSerProAlaSerLeuSerValSerVal

        130                            150                            170
GGAGAAACTGTCACCATCACATGTCGAGCAAGTGAGAATATTTACAGTAATTTAGCATGG
GlyGluThrValThrIleThrCysArgAlaSerGluAsnIleTyrSerAsnLeuAlaTrp

        190                            210                            230
TATCAACAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATGCTGCAACAAACTTAGCA
TyrGlnGlnLysGlnGlyLysSerProGlnLeuLeuValTyrAlaAlaThrAsnLeuAla

        250                            270                            290
GATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCGGGCACACAGTATTCCCTCAAGATC
AspGlyValProSerArgPheSerGlySerGlySerGlyThrGlnTyrSerLeuLysIle

        310                            330                            350
AACAGCCTGCAGTCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTTGGGGTACTCCG
AsnSerLeuGlnSerGluAspPheGlySerTyrTyrCysGlnHisPheTrpGlyThrPro

        370                            390                            410     ▼
TACACGTTCGGAGGGGGGACCAGGCTGGAAATAAAACGGGCTGATGCTGCACCAACTGTC
TyrThrPheGlyGlyGlyThrArgLeuGluIleLysArgAlaAspAlaAlaProThrVal
```

FIG. 2

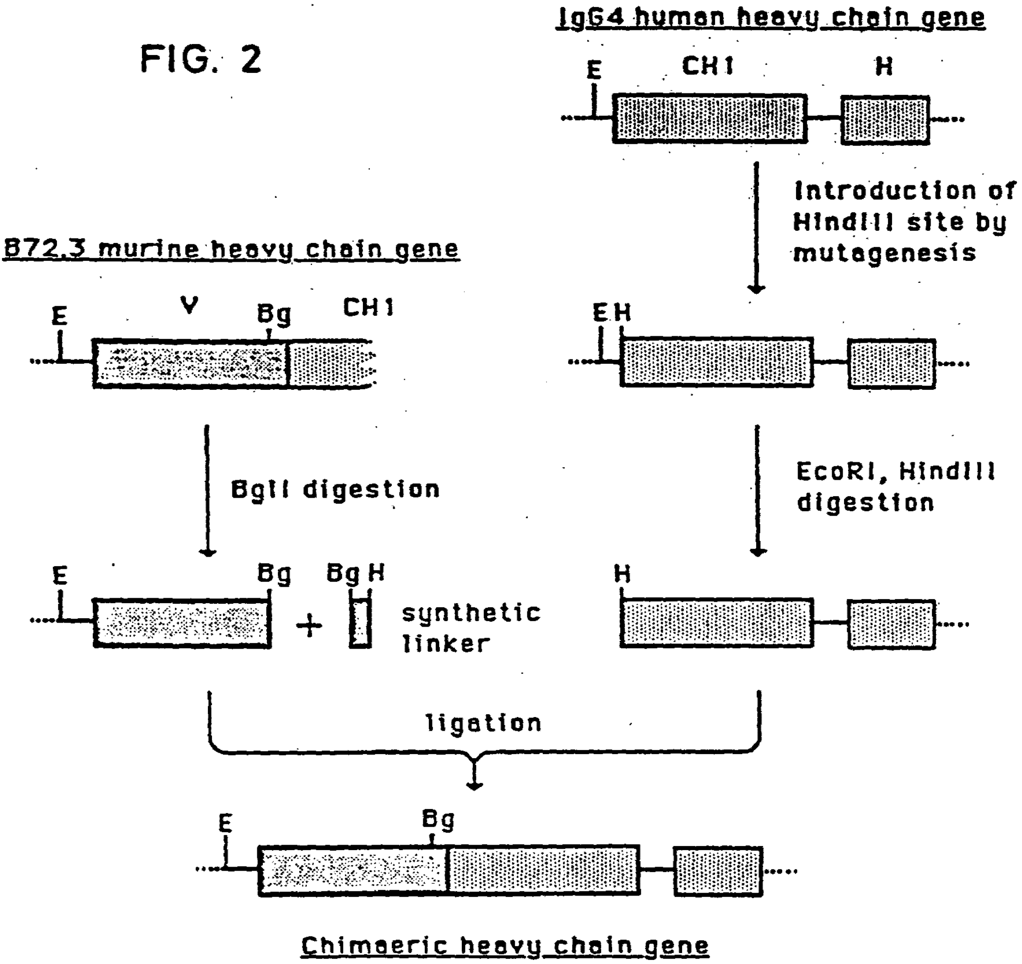
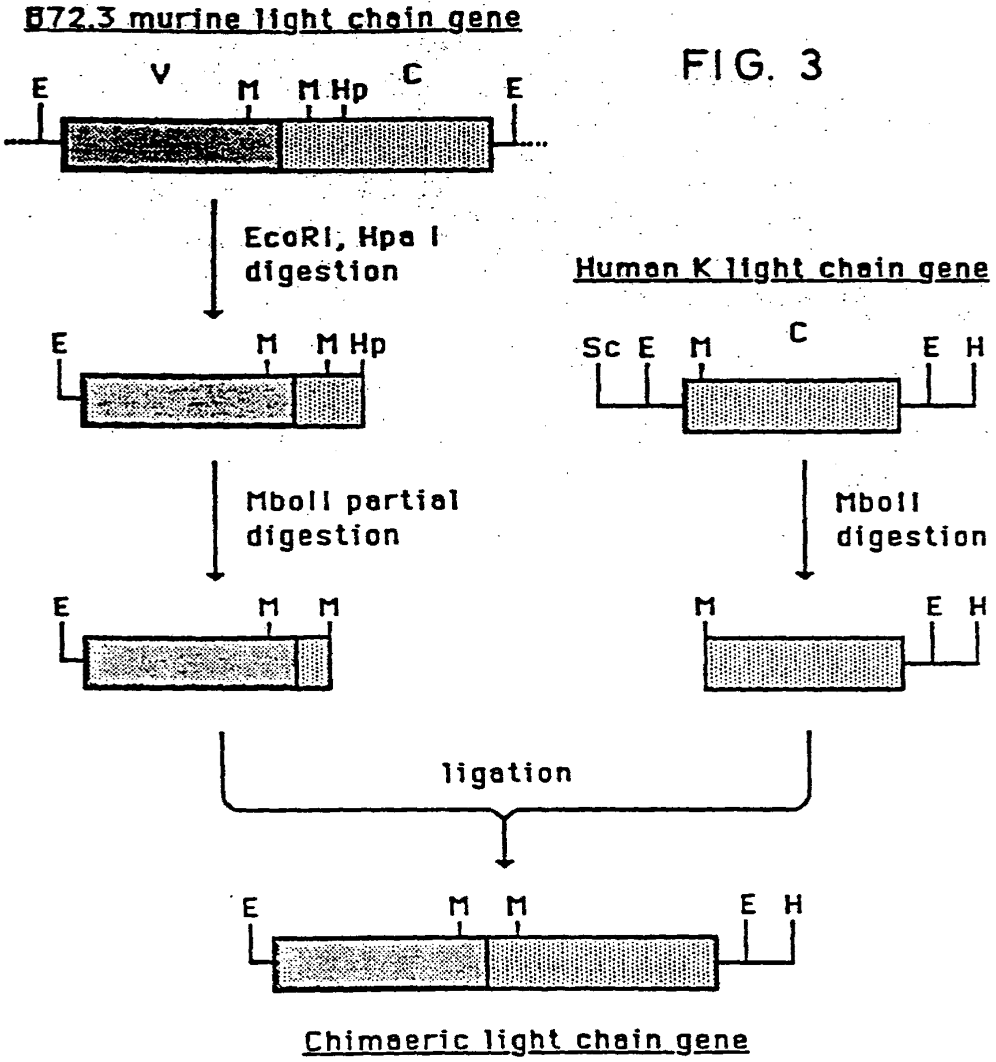

IgG4 human heavy chain gene
E
CH1
H
Introduction of HindIII site by mutagenesis
B72.3 murine heavy chain gene
E
V
Bg
CH1
E H
BglI digestion
EcoRI, HindIII digestion
E
Bg
Bg H
+
synthetic linker
H
ligation
E
Bg
Chimaeric heavy chain gene

FIG. 3


B72.3 murine light chain gene
E
V
M
M Hp
C
E
EcoRI, Hpa I
digestion
E
M
M Hp
Human K light chain gene
Sc E
M
C
E H
MboII partial
digestion
MboII
digestion
E
M
M
M
E H
ligation
E
M
M
E H
Chimaeric light chain gene

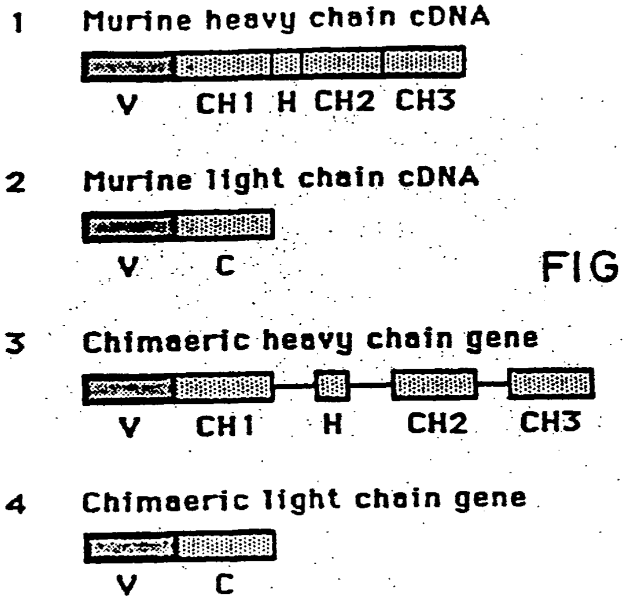
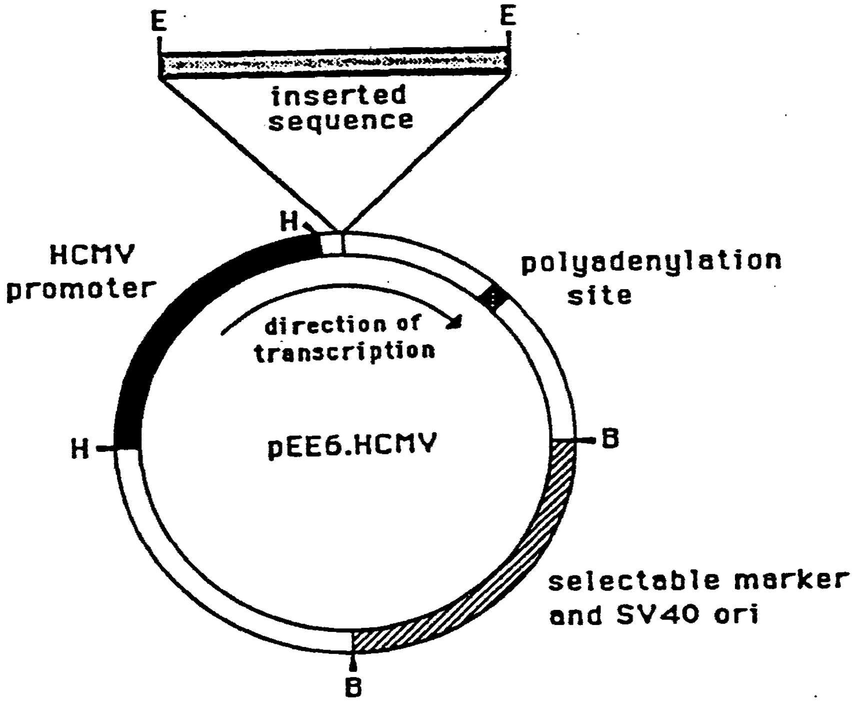


FIG. 4



SUBSTITUTE SHEET

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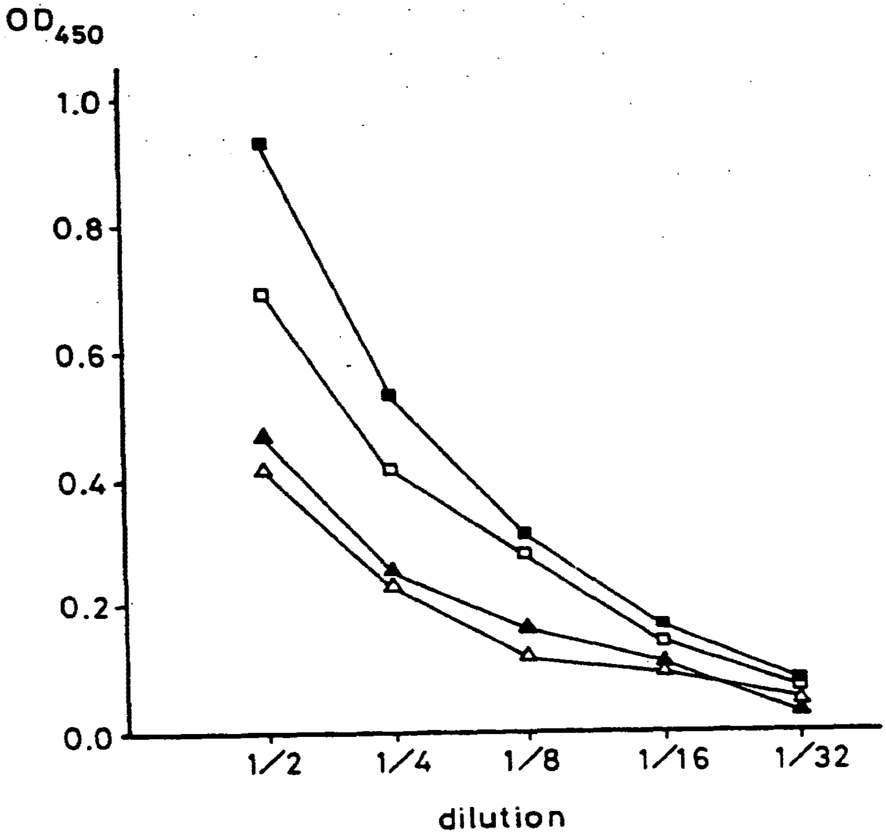
FIG. 5



SUBSTITUTE SHEET

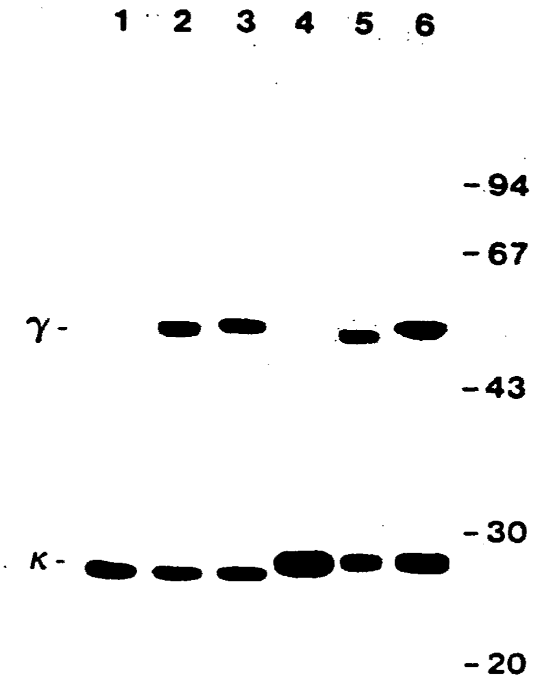


FIG. 6

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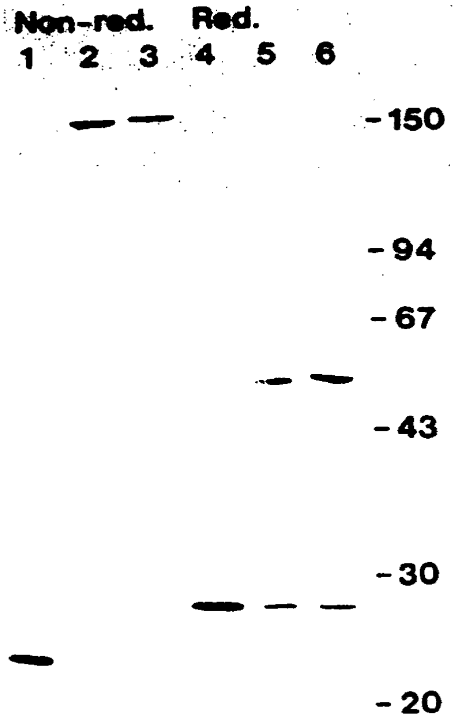


FIG. 7

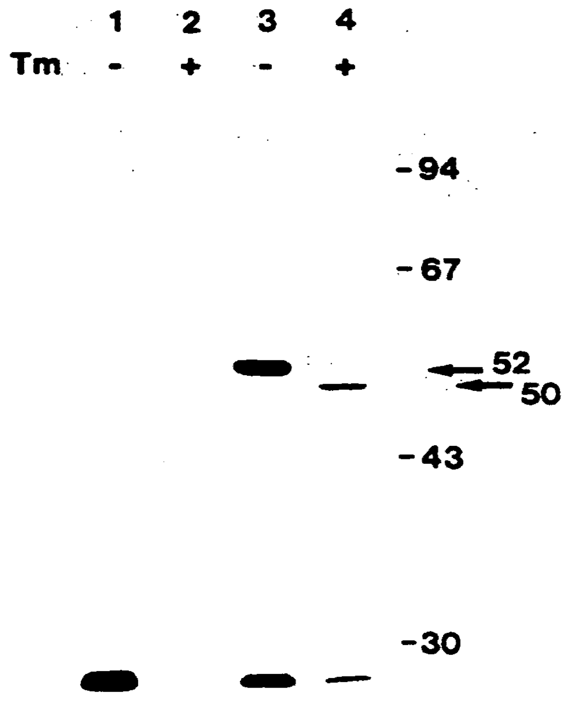


FIG. 8

SDS-PAGE OF CHIMAERIC B72.3

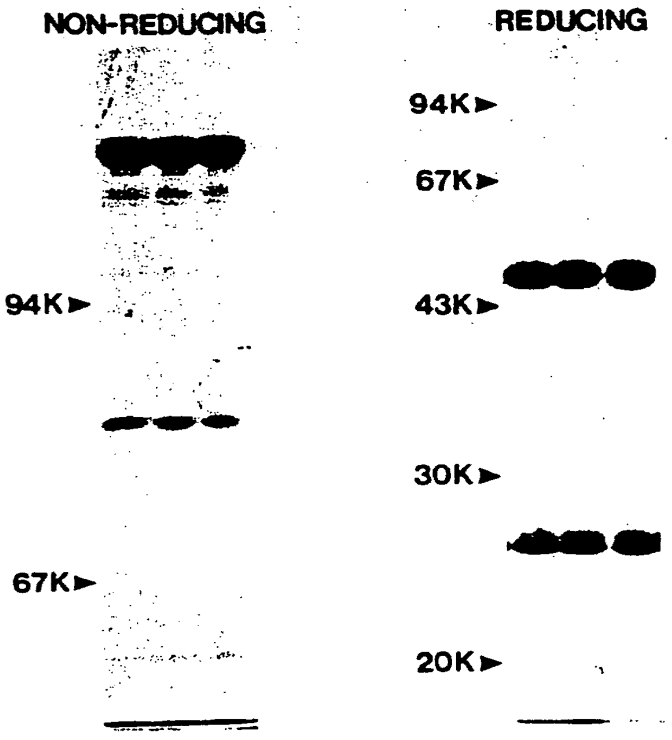
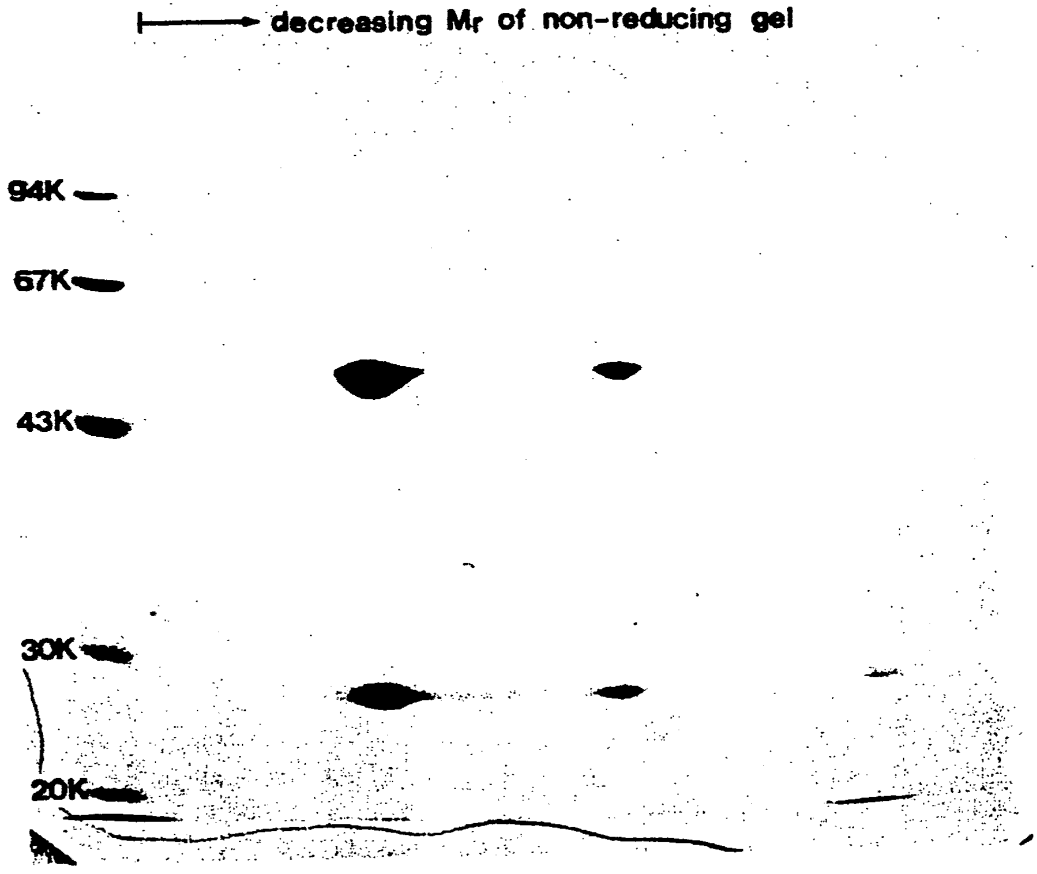


FIG. 9

10/13

2-DIMENSIONAL SDS-PAGE OF CHIMAERIC B72.3

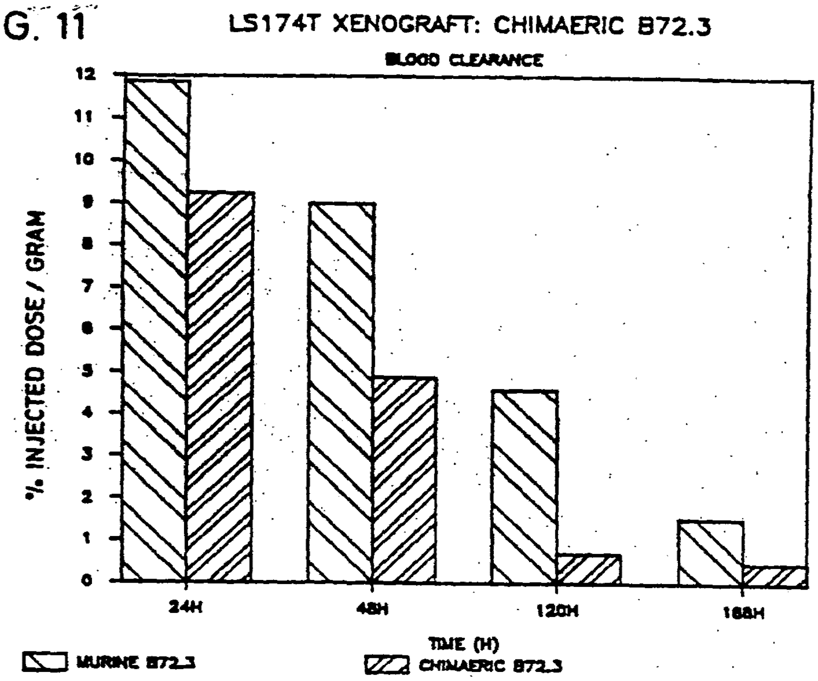


1st dimension: non-reducing SDS-PAGE
2nd dimension: reducing SDS-PAGE

FIG. 10

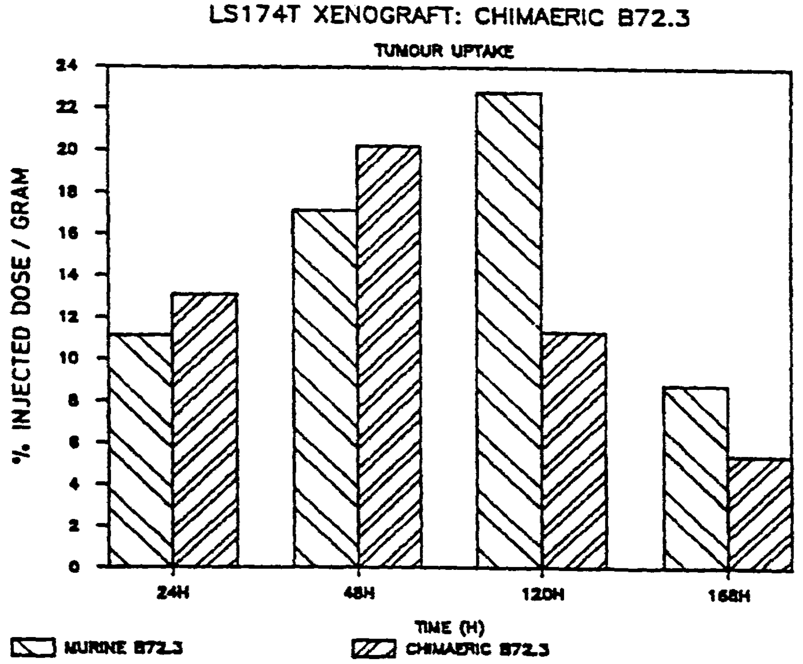
SUBSTITUTE SHEET

FIG. 11



LS174T XENOGRAFT: CHIMAERIC B72.3

BLOOD CLEARANCE



LS174T XENOGRAFT: CHIMAERIC B72.3

TUMOUR UPTAKE

SUBSTITUTE SHEET

FIG. 12

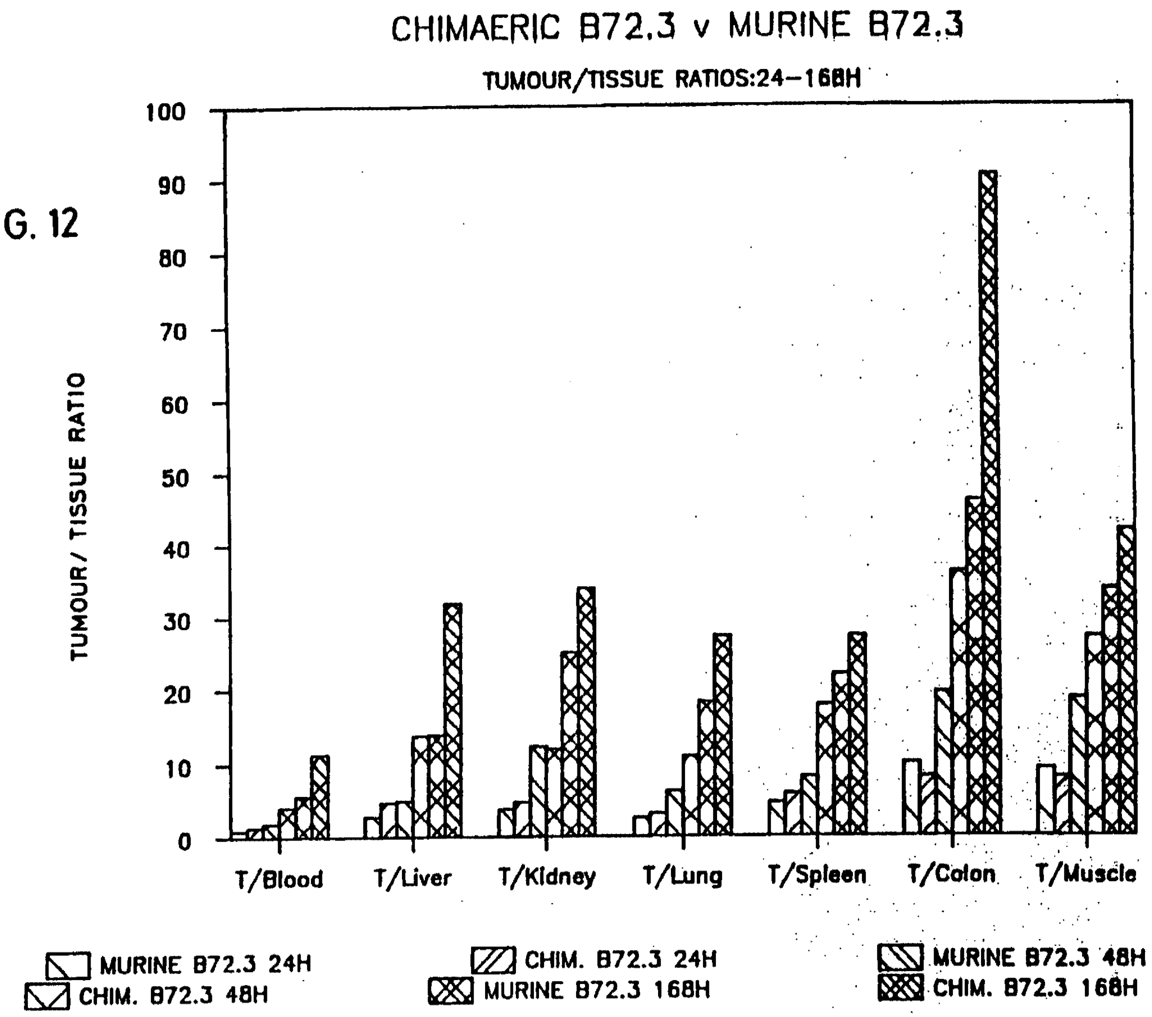
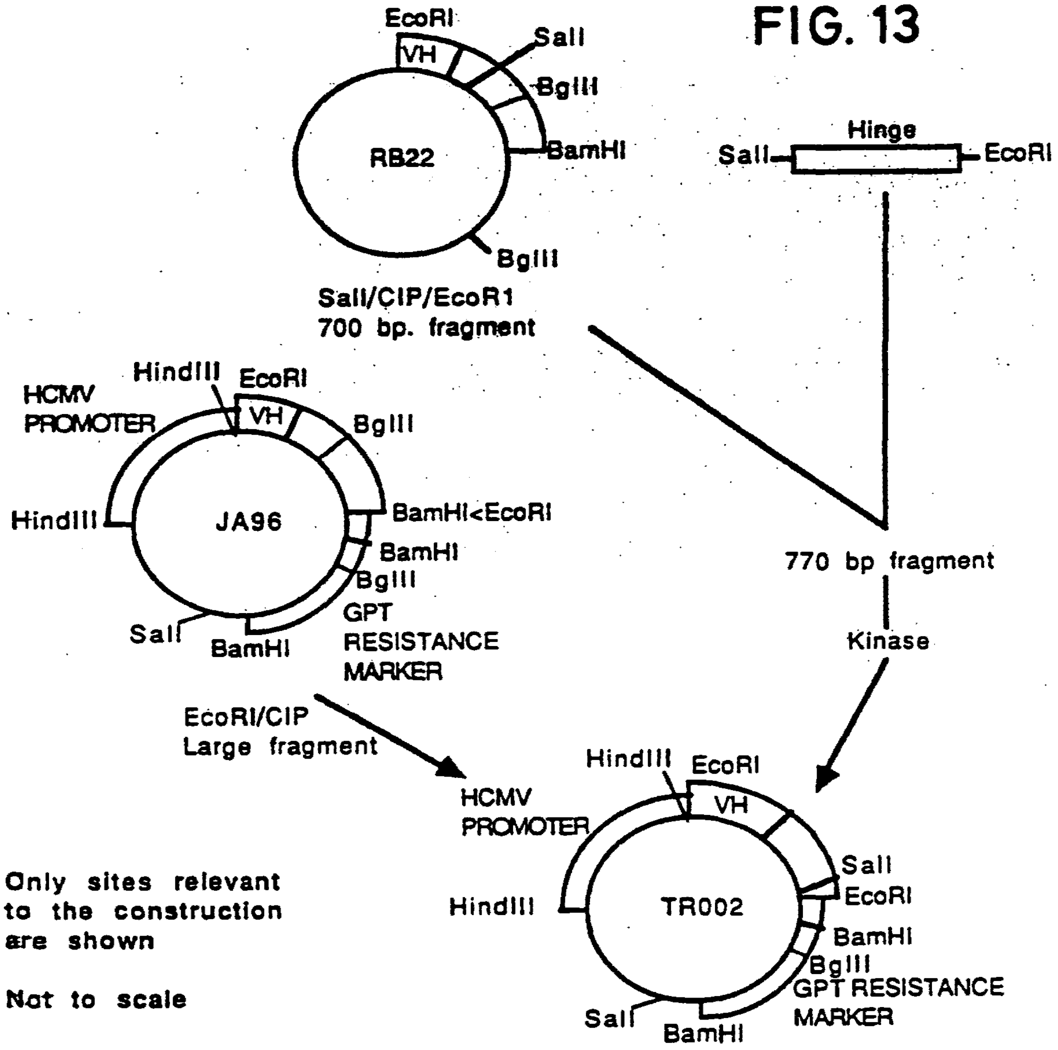

CHIMAERIC B72.3 v MURINE B72.3
TUMOUR/TISSUE RATIOS:24–168H
TUMOUR / TISSUE RATIO
100
90
80
70
60
50
40
30
20
10
0
T/Blood
T/Liver
T/Kidney
T/Lung
T/Spleen
T/Colon
T/Muscle
MURINE B72.3 24H
CHIM. B72.3 48H
CHIM. B72.3 24H
MURINE B72.3 168H
MURINE B72.3 48H
CHIM. B72.3 168H

FIG. 13



Only sites relevant to the construction are shown

Not to scale

CONSTRUCTION OF TR002
B72.3VH/HUMAN IgG1 F(ab') CONSTANT
cDNA LIKE CHIMAERIC HEAVY CHAIN (γ1 HINGE)
IN EE6 BASED EXPRESSION VECTOR

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
C12P 21/00, C12N 15/00, 5/00
G01N 33/574, A61K 39/395

A1

(11) International Publication Number: **WO 89/ 06692**

(43) International Publication Date: 27 July 1989 (27.07.89)

(21) International Application Number: PCT/US89/00051

(22) International Filing Date: 5 January 1989 (05.01.89)

(31) Priority Application Numbers: 143,912
147,461

(32) Priority Dates: 12 January 1988 (12.01.88)
25 January 1988 (25.01.88)

(33) Priority Country: US

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(81) Designated State: JP.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD OF TREATING TUMOR CELLS BY INHIBITING GROWTH FACTOR RECEPTOR FUNCTION

(57) Abstract

A method of inhibiting growth of tumor cells which overexpress a growth factor receptor or growth factor by treatment of the cells with antibodies which inhibit the growth factor receptor function is disclosed. A method of treating tumor cells with antibodies which inhibit growth factor receptor function, and with cytotoxic factor(s) such as tumor necrosis factor, is also disclosed. By inhibiting growth factor receptor functions tumor cells are rendered more susceptible to cytotoxic factors.

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Carter et al. P0709P1
SN: 08/146,206
Filed November 17, 1993

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METHOD OF TREATING TUMOR CELLS BY INHIBITING GROWTH FACTOR RECEPTOR FUNCTION

Field of the Invention

This invention is in the fields of immunology and cancer diagnosis and therapy. More particularly it concerns antibodies specifically binding growth factor receptors, hybridomas that produce these antibodies, immunochemicals made from the antibodies, and diagnostic methods that use the antibodies. The invention also relates to the use of the antibodies alone or in combination with cytotoxic factor(s) in therapeutic methods. Also encompassed by the invention is an assay for tyrosine kinases that are involved in tumorigenesis.

Background of the Invention

Macrophages are one of the effector cell types that play an important role in immunosurveillance against neoplastic growth in vivo. In vitro, cell-mediated cytotoxicity requires selective binding between activated macrophages and target cells as well as the concomitant release of cytotoxic factors. Some of the cytotoxic factors secreted by activated macrophages include reactive oxygen species such as the superoxide anion and hydrogen peroxide, arginase, interleukin 1, and tumor necrosis factor-α (TNF-α). Acquired resistance to the toxic effects of these factors by tumor cells could be one mechanism which leads to the onset and spread of tumor formation in vivo.

The observation that TNF-α can act as a potent effector of the macrophage-mediated antitumor response provides a rationale for its use in further studies on the regulation of tumorigenesis in vivo and tumor cell growth in vitro. The genes encoding TNF-α and TNF-β, a structurally related cytotoxic protein formerly known as lymphotoxin, have been cloned and the corresponding proteins expressed in *Escherichia coli*. These proteins display an array of biological activities, including induction of hemorrhagic necrosis of Meth A sarcomas in vivo, inhibition of the growth of certain tumor cells in vitro, synergistic enhancement of the in vitro anticellular effects of IFN-γ, activation of human polymorphonuclear neutrophil functions, and inhibition of lipid biosynthesis. Recently, rHuTNF-α was shown to augment the growth of normal diploid fibroblasts in vitro. The divergent proliferative responses in the presence of rHuTNF-α are sometimes related to variations in TNF binding.

Growth factors and their receptors are involved in the regulation of cell proliferation and they also appear to play a key role in oncogenesis. Of the known proto-oncogenes, three are related to a growth factor or a growth factor receptor. These genes include *c-sis*, which is homologous to the transforming gene of the simian sarcoma virus and is the B chain of platelet-derived growth factor (PDGF); *c-fms*, which is homologous to the transforming gene of the feline sarcoma virus and is closely related to the macrophage colony-stimulating factor receptor (CSF-1R); an *c-erb*B, which encodes the EGF receptor (EGFR) and is homologous to the transforming gene of the avian erythroblastosis virus (*v-erb*B). The two receptor-related proto-oncogenes, *c-fms* and *c-erb*B, are members of the tyrosine-specific protein kinase family to which many proto-oncogenes belong.

Recently, a novel transforming gene was identified as a result of transfection studies with DNA from chemically induced rat neuroblastomas. This gene, called *neu*, was shown to be related to, but distinct from, the *c-erb*B proto-oncogene. By means of *v-erb*B and human EGFR as probes to screen human genomic and complementary DNA (cDNA) libraries, two other groups independently isolated human *erb*B-related genes that they called HER2 and *c-erb*B-2 respectively. Subsequent sequence analysis and chromosomal mapping studies revealed that *c-erb*B-2, and HER2 are species variants of *neu*. A fourth group, also using *v-erb*B as a probe, identified the same gene in a mammary carcinoma cell line, MAC 117, where it was found to be amplified five- to ten-fold.

This gene, which will be referred to herein as HER2, encodes a new member of the tyrosine kinase family; and is closely related to, but distinct from, the EGFR gene as reported by Coussens _et al_., _Science_ _230_, 1132 (1985). HER2 differs from EGFR in that it is found on band q21 of chromosome 17, as compared to band p11-p13 of chromosome 7; where the EGFR gene is located. Also, the HER2 gene generates a messenger RNA (mRNA) of 4.8 kb, which differs from the 5.8- and 10-kb transcripts for the EGFR gene. Finally, the protein encoded by the HER2 gene is 185,000 daltons, as compared to the 170,000-dalton protein encoded by the EGFR gene. Conversely, on the basis of sequence data, HER2 is more closely related to the EGFR gene than to other members of the tyrosine kinase family. Like the EGFR protein, the HER2 protein (p185) has an extracellular domain, a transmembrane domain that includes two cysteine-rich repeat clusters, and an intracellular kinase domain, indicating that it is likely to be a cellular receptor for an as yet unidentified ligand. HER2 p185 is referred to as p185 or the HER2 receptor herein.

Southern analysis of primary human tumors and established tumor-derived cell lines revealed amplification and in some cases rearrangement of the EGF receptor gene. Amplification was particularly apparent in squamous carcinomas and glioblastomas. The HER2 gene was also found to be amplified in a human salivary gland adenocarcinoma, a renal adenocarcinoma, a mammary gland carcinoma, and a gastric cancer cell line. Recently, Slamon _et al_., _Science_ _235_, 177 (1987) demonstrated that about 30% of primary human breast carcinoma tumors contained an amplified HER2 gene. Although a few sequence rearrangements were detected, in most tumors there were no obvious differences between amplified and normal HER2 genes. Furthermore, amplification of the HER2 gene correlated significantly with the negative prognosis of the disease and the probability of relapse.

To investigate the significance of the correlation between over-expression and cellular transformation as it has been observed for proto-oncogenes *c-mos* and *N-myc*, a HER2 expression vector and a selection scheme that permitted sequence amplification after transfection of mouse NIH 3T3 cells was employed by Hudziak et al., Proc. Natl. Acad. Sci. (USA) 84, 7159 (1987). Amplification of the unaltered HER2 gene in NIH 3T3 cells lead to over-expression of p185 as well as cellular transformation and tumor formation in athymic mice.

The effects of antibodies specifically binding growth factors or growth factor receptors has been studied. Examples are discussed below.

Rosenthal et al., Cell 46, 301 (1986) introduced a human TGF-α cDNA expression vector into established non-transformed rat fibroblast cells. Synthesis and secretion of TGF-α by these cells resulted in loss of anchored-dependent growth and induced tumor formation in nude mice. Anti-human TGF-α monoclonal antibodies prevented the rat cells from forming colonies in soft agar, i.e. loss of anchorage dependence. Gill et al., in J. Biol. Chem. 259 7755 (1984) disclose monoclonal antibodies specific for EGF receptor which were inhibitors of EGF binding and antagonists of EGF-stimulated tyrosine protein kinase activity.

Drebin et al, in Cell 41 695 (1985) demonstrated that exposure of a *neu*-oncogene-transformed NIH 3T3 cell to monoclonal antibodies reactive with the *neu* gene product, cause the *neu*-transformed NIH 3T3 cell to revert to a non-transformed phenotype as determined by anchorage independent growth. Drebin et al., in Proc. Natl. Acad. Sci. 83, 9129 (1986) demonstrated that in vivo treatment with a monoclonal antibody (IgG2a isotype) specifically binding the protein encoded by the *neu* oncogene significantly inhibited the tumorigenic growth of *neu*-transformed NIH 3T3 cells implanted into nude mice.

Akiyama et al. in Science 232, 1644 (1986) raised antibodies against a synthetic peptide corresponding to 14 amino acid residues at the carboxy-terminus of the protein deduced from the *c-erb*B-2 (HER2) nucleotide sequence.

Growth factors have been reported to interact in both a synergistic and an antagonistic manner. For example, TGF-α and TGF-β synergistically enhance the growth of NRK-49F fibroblasts, whereas PDGF down regulates EGF receptor function on 3T3 cells. A variety of transformed cells secrete factors which are believed to stimulate growth by an autocrine mechanism. Sugarman et al., Cancer Res. 47, 780 (1987) demonstrated that under certain conditions, growth factors can block the antiproliferative effects of TNF-α on sensitive tumor cells. Specifically, epidermal growth factor (EGF) and recombinant human transforming growth factor-α (rHuTGF-α) were shown to interfere with the in vitro antiproliferative effects of recombinant human tumor necrosis factor-α (rHuTNF-α) and -β on a human cervical carcinoma cell line, ME-180. The inhibitory effect could be observed at EGF or rHuTGF-α concentrations of 0.1 to 100 ng/ml, and was maximal between 1 and 10 ng/ml. This response was apparently not due to down regulation of the TNF receptor or to alteration of the affinity of TNF-α for its receptor. Since the antiproliferative effect of recombinant human interferon-γ was not significantly affected by the presence of EGF or rHuTGF-α, the inhibition was specific for recombinant TNFs and was not due solely to enhanced proliferation induced by the growth factors. Neither growth factor had a substantial protective effect on the synergistic cytotoxicity observed when tumor cells were exposed simultaneously to rHuTNF-α and recombinant human interferon-γ. TGF-β can also interfere with the antiproliferative effects of rHuTNF-α in vitro. At concentrations of less than 1 ng/ml, TGF-β significantly antagonized the cytotoxic effects of rHuTNF-α on NIH 3T3 fibroblasts. Since EGF, platelet-derived growth factor, and TGF-β all enhanced NIH 3T3 cell proliferation, but only TGF-β interfered with rHuTNF-α cytotoxicity, the protective effects of TGF-β were not related in a simple manner to enhanced cell proliferation. rHuTGF-α and TGF-β did not have a significant protective effect against rHuTNF-α-mediated cytotoxicity on two other tumor cell lines, BT-20 and L-929 cells.

It is an object of the subject invention to provide antibodies capable of inhibiting growth factor receptor function.

It is a further object of the invention to provide an improved assay for the HER2 receptor.

It is a further object of the invention to provide improved methods of tumor therapy.

It is a further object of the invention to provide a method of inhibiting the growth of tumor cells which overexpress a growth factor receptor and/or growth factor.

It is a further object of the invention to provide a method for treating a tumor by treatment of the tumor cells with antibodies capable of inhibiting growth factor receptor function, and with cytotoxic factors such as tumor necrosis factor.

A still further object of the invention is to provide an assay for tyrosine kinases that may have a role in tumorigenesis.

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

Summary of the Invention

The subject invention relates to monoclonal antibodies specifically binding the external domain of the HER2 receptor. The invention also relates to an assay for the HER2 receptor comprising exposing cells to antibodies specifically binding the extracellular domain of the HER2 receptor, and determining the extent of binding of said antibodies to said cells. Another embodiment of the invention relates to a method of inhibiting growth of tumor cells by administering to a patient a therapeutically effective amount of antibodies capable of inhibiting the HER2 receptor function. A further embodiment of the invention relates to administering a therapeutically effective amount of antibodies capable of inhibiting growth factor receptor function, and a therapeutically effective amount of a cytotoxic factor. A still further embodiment of the invention is an assay for tyrosine kinases that may have a role in tumorigenesis comprising exposing cells suspected to be TNF-α resistant to TNF-α, isolating those cell which are TNF-α resistant, screening the isolated cells for increased tyrosine kinase activity, and isolating receptors and other proteins having increased tyrosine kinase activity.

Brief Description of the Drawings

Figure 1a shows TNF-α resistance of NIH 3T3 cells expressing various levels of HER2 p185. Figure 1b shows macrophage cytotoxicity assays for NIH 3T3 cells expressing various levels of HER2 p185.

Figure 2 demonstrates the level of TNF-α binding for a control cell line (NIH 3T3 neo/dhfr) and for a cell line overexpressing HER2 p185 (HER2-3_{800}).

Figure 3 shows inhibition of SK BR3 cell growth by anti-HER2 monoclonal antibodies.

Figure 4 is a dose response curve comparing the effect of an irrelevant monoclonal antibody (anti-HBV) and the effect of monoclonal antibody 4D5 (anti-HER2) on the growth of SK BR3 cells in serum.

Figures 5a, 5b and 6a show percent viability of SK BR3 cells as a function of increasing TNF-α concentration and anti-HER2 p185 monoclonal antibody concentration. Each Figure shows the results for a different anti-HER2 p185 monoclonal antibody. Figure 6b is a control using an irrelevant monoclonal antibody.

Figure 7 shows percent viability of MDA-MB-175-VII cells as a function of increasing TNF-α concentration and anti-HER2 p185 monoclonal antibody concentration.

Figure 8 shows percent viability of NIH 3T3 cells overexpressing HER2 p185 as a function of increasing TNF-α concentration and anti-HER2 p185 monoclonal antibody concentration.

Detailed Description of the Invention

A new application of antibodies to inhibit the growth of tumor cells has been discovered. Surprisingly, it has been found that by inhibiting growth factor receptor function, e.g. the HER2 receptor function, cell growth is inhibited, and the cells are rendered more susceptible to cytotoxic factors. Thus, for example, breast cancer cells which are refractory to TNF-α alone can be made susceptible to TNF-α if the cells are first treated with antibodies which inhibit growth factor receptor function. The increase of susceptibility has been demonstrated using the HER2 receptor and monoclonal antibodies directed against the HER2 receptor, and tumor necrosis factor-α.

The method of this invention is useful in the therapy of malignant or benign tumors of mammals where the abnormal growth rate of the tumor is dependent upon growth factor receptors.

Abnormal growth rate is a rate of growth which is in excess of that required for normal homeostasis and is in excess of that for normal tissues of the same origin. Many of these tumors are dependent upon extracellular sources of the growth factor recognized by the receptor, or upon synthesis of the growth factor by the tumor cell itself. This latter phenomenon is termed "autocrine" growth.

The methods of the subject invention is applicable where the following conditions are met:

(1) the growth factor receptor and/or ligand (growth factor) is expressed, and tumor cell growth depends upon the growth factor receptor biological function;

(2) antibodies specifically binding the growth factor receptor and/or ligand inhibit the growth factor receptor biological function.

While not wishing to be constrained to any particular theory of operation of the invention, it is believed that the antibodies inhibit growth factor receptor biological function in one or more of the following ways:

(a) The antibodies bind to the extracellular domain of the receptor and inhibit the ligand from binding the receptor;

(b) The antibodies bind the ligand (the growth factor) itself and inhibit the ligand from binding the receptor;

(c) The antibodies down regulate the growth factor receptor;

(d) The antibodies sensitize tumor cells to the cytotoxic effects of a cytotoxic factor such as TNF-α;

(e) The antibodies inhibit the tyrosine kinase activity of the receptor.

In cases (f) and (g), the antibodies inhibit growth factor receptor biological function indirectly by mediating cytotoxicity via a targeting function:

(f) The antibodies belong to a sub-class or isotype that upon complexing with the receptor activates serum complement and/or mediate antibody-dependent cellular cytotoxicity (ADCC), e.g. IgG2a antibodies;

(g) The antibodies which bind the receptor or growth factor are conjugated to a toxin (immunotoxins);

Advantageously antibodies are selected which greatly inhibit the receptor function by binding the steric vicinity of the ligand binding site of the receptor (blocking the receptor), and/or which bind the growth factor in such a way as to prevent (block) the ligand from binding to the receptor. These antibodies are selected using conventional <u>in vitro</u> assays for selecting antibodies which neutralize receptor function. Antibodies that act as ligand agonists by mimicking the ligand are discarded by conducting suitable assays as will be apparent to those skilled in the art. For certain tumor cells, the antibodies inhibit an autocrine growth cycle (i.e. where a cell secretes a growth factor which then binds to a receptor of the same cell). Since some ligands, e.g. TGF-α, are found lodged in cell membranes, the antibodies serving a targeting function are directed against the ligand and/or the receptor.

Certain tumor cells secrete growth factors that are required for normal cellular growth and division. These growth factors, however, can under some conditions stimulate unregulated growth of the tumor cell itself, as well as adjacent non-tumor cells, and can cause a tumor to form.

Epidermal Growth Factor (EGF) has dramatic stimulatory effects on cell growth. In purified receptor preparations, the EGF receptor is a protein kinase that is activated by the binding of EGF. Substrate proteins for this kinase are phosphorylated on tyrosine residues. The receptors for insulin, platelet-derived growth factor (PDGF) and other growth hormones also are tyrosine-specific kinases. It is believed that ligand binding to the receptor triggers phosphorylation of certain proteins by the receptor and in this way stimulates cell growth. About one-third of the known oncogenes encode proteins that phosphorylate tyrosine residues on other proteins. It is believed that these oncogene products trigger responses analogous to the responses of cells to growth factors and hormones. The *erb*B oncogene product is a portion of the EGF receptor that lacks the hormone-binding domain and may give rise to a constitutive growth-stimulating signal.

One embodiment of this invention is a method of inhibiting the growth of tumor cells by administering to a patient a therapeutically effective amount of antibodies that inhibit the HER2 receptor biological function of tumor cells.

Overexpression of growth factor receptors increases the resistance of cells to TNF as demonstrated below. Overexpression of the HER1 receptor (EGF receptor), met receptor-like protooncogene product, and HER2 receptor all show this increased resistance. It is shown in the Examples below that amplified expression of HER2, which encodes the HER2 receptor (p185), induces resistance of NIH 3T3 cells to the cytotoxic effects of macrophages or TNF-α. Induction of NIH 3T3 cell resistance to TNF-α by overexpression of p185 is accompanied by alterations in the binding of TNF-α to its receptor. Overexpression of p185 is also associated with resistance of certain human breast tumor cell lines to the cytotoxic effects of TNF-α.

In another embodiment of the invention, tumor cells are treated by (1) administering to a patient antibodies directed against the growth factor and/or its receptor, that inhibit the biological function of the receptor and that sensitize the cells to cytotoxic factors such as TNF, and (2) administering to the patient cytotoxic factor(s) or other biological response modifiers which activate immune system cells directly or indirectly to produce cytotoxic factors.

The cytotoxic factor, such as TNF-α, exerts its cytostatic (cell growth suppressive) and cytotoxic (cell destructive) effect. Examples of useful cytotoxic factors are TNF-α, TNF-β, IL-1, IFN-γ and IL-2, and chemotherapeutic drugs such as 5FU, vinblastine, actinomycin D, etoposide, cisplatin, methotrexate, and doxorubicin. Cytotoxic factors can be administered alone or in combination. In a still further embodiment of the invention, the patient is treated with antibodies which inhibit receptor function, and with autologous transfer therapy, e.g. LAK or TIL cells.

Tumor necrosis factors are polypeptides produced by mitogen-stimulated macrophages or lymphocytes which are cytotoxic for certain malignantly transformed cells. The anti-tumor effect of TNF-α is known to be synergistically potentiated by interferons. The anti-tumor effect of TNF-α and TNF-β in admixture are additive, as are the antiviral effects of interferons alpha and beta.

The tumor necrosis factors include TNF-α and TNF-β. The former is described together with methods for its synthesis in recombinant cell culture, in U.S. Patent 4,650,674, and in European Patent Application 0168214; the latter is described in European Patent Application 0164965. The TNF-α and TNF-β described in these patent documents includes cytotoxic amino acid sequence and glycosylation variants. TNF-α and TNF-β from non-recombinant sources are also useful in the method of this invention.

The preferred TNF is mature human TNF-α from recombinant microbial cell culture. The TNF ordinarily will have a cytolytic activity on susceptible L-M murine cells of greater than about 1 x 10^6 units/mg, wherein a unit is defined as set forth in the above-described patent application.

In another embodiment of the subject invention, one or more additional cytokines and/or cytotoxic factors are administered with TNF-α, egs. interferons, interleukins, and chemotherapeutic drugs.

The compositions herein include a pharmaceutically acceptable vehicle such as those heretofore used in the therapeutic administration of interferons or TNF, e.g. physiological saline or 5% dextrose, together with conventional stabilizers and/or excipients such as human serum albumin or mannitol. The compositions are provided lyophilized or in the form of sterile aqueous solutions.

Several variables will be taken into account by the ordinary artisan in determining the concentration of TNF in the therapeutic compositions and the dosages to be administered. Therapeutic variables also include the administration route, and the clinical condition of the patient.

The cytotoxic factor(s) and antibodies inhibiting growth factor receptor function are administered together or separately. If the latter, advantageously the antibodies are administered first and the TNF thereafter within 24 hours. It is within the scope of this invention to administer the TNF and antibodies in multiple cycles, depending upon the clinical response of the patient. The TNF and antibodies are administered by the same or separate routes, for example by intravenous, intranasal or intramuscular administration.

The method of the subject invention can be used with tumor cells which overexpress growth factor receptor and/or ligand where antibodies can be produced which inhibit the growth factor receptor function. A cell (e.g. breast tumor cell) overexpresses a growth factor receptor if the number of receptors on the cell exceeds the number on the normal healthy cell (e.g. normal breast tissue cell). Examples of carcinomas where the HER2 receptor is overexpressed (and thus the method of the subject invention is applicable), are human breast, renal, gastric and salivary gland carcinomas.

A further embodiment of the invention is an assay for identifying receptors and other proteins having increased tyrosine kinase activity, and for identifying oncogenes that transform cells. Amplification of certain oncogenes encoding tyrosine kinases correlates with TNF-α resistance. If cells are selected for resistance to TNF-α, some of these will have increased tyrosine kinase activity. Some of the tyrosine kinases will be receptors. The genes encoding the tyrosine kinases are then cloned using standard techniques for the cloning of genes. Identification of the receptor or other protein permits the design of reagents which inhibit receptor (or other protein) function and induce cellular sensitivity to cytotoxic factors as demonstrated herein with HER2. Identification of the receptor also permits subsequent identification of the receptor's ligand. The assay comprises exposing cells suspected to be TNF-α sensitive to TNF-α, and isolating those cells which are TNF-α resistant. The TNF-α resistant cells are then screened for increased tyrosine kinase activity, and receptors and other proteins having increased tyrosine kinase activity are isolated.

Antibodies

In accordance with this invention, monoclonal antibodies specifically binding growth factors or growth factor receptors such as the HER2 receptor, were isolated from continuous hybrid cell lines formed by the fusion of antigen-primed immune lymphocytes with myeloma cells. Advantageously, the monoclonal antibodies of the subject invention which bind growth factor receptors, bind the extracellular domain of the receptors. In another embodiment of the invention, polyclonal antibodies specifically binding the growth factors or growth factor receptors are used.

The antibodies of the subject invention which are used in tumor therapy advantageously inhibit tumor cell growth greater than 20%, and most advantageously greater than 50%, <u>in vitro</u>. These antibodies are obtained through screening (see, for example, the discussion relating to Figure 3). The anti-HER2 receptor monoclonal antibodies of the subject invention which are used in tumor therapy are capable of inhibiting serum activation of the receptor.

Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional antibody (polyclonal) preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. Monoclonal antibodies are useful to improve the selectivity and specificity of diagnostic and analytical assay methods using antigen-antibody binding. A second advantage of monoclonal antibodies is that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may be prepared from supernatants of cultured hybridoma cells or from ascites induced by intra-peritoneal inoculation of hybridoma cells into mice.

The hybridoma technique described originally by Kohler and Milstein, Eur. J. Immunol. 6, 511 (1976) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

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

After immunization, immune lymphoid cells are fused with myeloma cells to generate a hybrid cell line which can be cultivated and subcultivated indefinitely, to produce large quantities of monoclonal antibodies. For purposes of this invention, the immune lymphoid cells selected for fusion are lymphocytes and their normal differentiated progeny, taken either from lymph node tissue or spleen tissue from immunized animals. Applicants prefer to employ immune spleen cells, since they offer a more concentrated and convenient source of antibody producing cells with respect to the mouse system. The myeloma cells provide the basis for continuous propagation of the fused hybrid. Myeloma cells are tumor cells derived from plasma cells.

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

The hybrid cell lines can be maintained in culture _in vitro_ in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in the known hypoxanthine-aminopterinthymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody. The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange chromatography, affinity chromatography, or the like.

While the invention is demonstrated using mouse monoclonal antibodies, the invention is not so limited; in fact, human antibodies may be used and may prove to be preferable. Such antibodies can be obtained by using human hybridomas (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985)). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al. Proc. Natl. Acad. Sci. 81, 6851 (1984); Neuberger et al., Nature 312, 604 (1984); Takeda et al., Nature 314, 452 (1985)) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity (such as ability to activate human complement and mediate ADCC) can be used; such antibodies are within the scope of this invention.

As another alternative to the cell fusion technique, EBV immortalized B cells are used to produce the monoclonal antibodies of the subject invention. Other methods for producing monoclonal antibodies such as recombinant DNA, are also contemplated.

The immunochemical derivatives of the antibodies of this invention that are of prime importance are (1) immunotoxins (conjugates of the antibody and a cytotoxic moiety) and (2) labeled (e.g. radiolabeled, enzyme-labeled, or fluorochrome-labeled) derivatives in which the label provides a means for identifying immune complexes that include the labeled antibody. The antibodies are also used to induce lysis through the natural complement process, and to interact with antibody dependent cytotoxic cells normally present.

Immunotoxins

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial or plant origin, or an enzymatically active fragment ("A chain") of such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruzinosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites* fordii proteins, dianthin proteins, Phytolacca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin. In another embodiment, the antibodies are conjugated to small molecule anticancer drugs. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such a dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

Advantageously, monoclonal antibodies specifically binding the external domain of the target growth factor receptor, e.g. HER2 receptor, are conjugated to ricin A chain. Most advantageously the ricin A chain is deglycosylated and produced through recombinant means. An advantageous method of making the ricin immunotoxin is described in Vitetta et al., Science 238, 1098 (1987).

When used to kill human cancer cells in vitro for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for in vitro use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques to determine the presence or degree of cancer.

Cytotoxic radiopharmaceuticals for treating cancer may be made by conjugating radioactive isotopes (e.g. I, Y, Pr) to the antibodies. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

In another embodiment, liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding a growth factor receptor. Since there are many receptor sites, this method permits delivery of large amounts of drug to the correct cell type.

Antibody Dependent Cellular Cytotoxicity

The present invention also involves a method based on the use of antibodies which are (a) directed against growth factor receptors such as HER2 p185, and (b) belong to a subclass or isotype that is capable of mediating the lysis of tumor cells to which the antibody molecule binds. More specifically, these antibodies should belong to a subclass or isotype that, upon complexing with growth factor receptors, activates serum complement and/or mediates antibody dependent cellular cytotoxicity (ADCC) by activating effector cells such as natural killer cells or macrophages.

The present invention is also directed to the use of these antibodies, in their native form, for therapy of human tumors. For example, many IgG2a and IgG3 mouse antibodies which bind tumor. associated cell surface antigens can be used *in vivo* for tumor therapy. In fact, since HER2 p185 is present on a variety of tumors, the subject antibodies and their therapeutic use have general applicability.

Biological activity of antibodies is known to be determined, to a large extent, by the Fc region of the antibody molecule (Uananue and Benacerraf, *Textbook of Immunology*, 2nd Edition, Williams & Wilkins, p. 218 (1984)). This includes their ability to activate complement and to mediate antibody-dependent cellular cytotoxicity (ADCC) as effected by leukocytes. Antibodies of different classes and subclasses differ in this respect, and, according to the present invention, antibodies of those classes having the desired biological activity are selected. For example, mouse immunoglobulins of the IgG3 and IgG2a class are capable of activating serum complement upon binding to the target cells which express the cognate antigen.

In general, antibodies of the IgG2a and IgG3 subclass and occasionally IgG1 can mediate ADCC, and antibodies of the IgG3, and IgG2a and IgM subclasses bind and activate serum complement. Complement activation generally requires the binding of at least two IgG molecules in close proximity on the target cell. However, the binding of only one IgM molecule activates serum complement.

The ability of any particular antibody to mediate lysis of the tumor cell target by complement activation and/or ADCC can be assayed. The tumor cells of interest are grown and labeled _in vivo_; the antibody is added to the tumor cell culture in combination with either serum complement or immune cells which may be activated by the antigen antibody complexes. Cytolysis of the target tumor cells is detected by the release of label from the lysed cells. In fact, antibodies can be screened using the patient's own serum as a source of complement and/or immune cells. The antibody that is capable of activating complement or mediating ADCC in the _in vitro_ test can then be used therapeutically in that particular patient.

Antibodies of virtually any origin can be used according to this embodiment of the present invention provided they bind growth factor receptors such as HER2 p185 _and_ can activate complement or mediate ADCC. Monoclonal antibodies offer the advantage of a continuous, ample supply. In fact, by immunizing mice with, for example, HER2 p185, establishing hybridomas making antibodies to p185 and selecting hybridomas making antibodies which can lyse tumor cells in the presence of human complement, it is possible to rapidly establish a panel of antibodies capable of reacting with and lysing a large variety of human tumors.

Therapeutic Uses of the Antibodies

When used in vivo for therapy, the antibodies of the subject invention are administered to the patient in therapeutically effective amounts (i.e. amounts that eliminate or reduce the patient's tumor burden). They will normally be administered parenterally, when possible, at the target cell site, or intravenously. The dose and dosage regimen will depend upon the nature of the cancer (primary or metastatic), its population, the site to which the antibodies are to be directed, the characteristics of the particular immunotoxin (when used), e.g., its therapeutic index, the patient, and the patient's history. The amount of antibody administered will typically be in the range of about 0.1 to about 10 mg/kg of patient weight.

For parenteral administration the antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic, and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The antibodies will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

The selection of an antibody subclass for therapy will depend upon the nature of the tumor antigen. For example, an IgM may be preferred in situations where the antigen is highly specific for the tumor target and rarely occurs on normal cells. However, where the tumor-associated antigen is also expressed in normal tissues, albeit at much lower levels, the IgG subclass may be preferred for the following reason: since the binding of at least two IgG molecules in close proximity is required to activate complement, less complement mediated damage may occur in the normal tissues which express smaller amounts of the antigen and, therefore, bind fewer IgG antibody molecules. Furthermore, IgG molecules by being smaller may be more able than IgM molecules to localize to tumor tissue.

There is evidence that complement activation in vivo leads to a variety of biological effects, including the induction of an inflammatory response and the activation of macrophages (Uananue and Benecerraf, Textbook of Immunology, 2nd Edition, Williams & Wilkins, p. 218 (1984)). Tumor cells are more sensitive to a cytolytic effect of activated macrophages than are normal cells, Fidler and Poste, Springer Semin. Immunopathol. 5, 161 (1982). The increased vasodilation accompanying inflammation may increase the ability of various anti-cancer agents, such as chemotherapeutic drugs, radiolabelled antibodies, etc., to localize in tumors. Therefore, antigen-antibody combinations of the type specified by this invention can be used therapeutically in many ways and may circumvent many of the problems normally caused by the heterogeneity of tumor cell populations. Additionally, purified antigens (Hakomori, Ann. Rev. Immunol. 2, 103 (1984)) or anti-idiotypic antibodies (Nepom et al., Proc. Natl. Acad. Sci. 81, 2864 (1985); Koprowski et al., Proc. Natl. Acad. Sci. 81, 216 (1984)) relating to such antigens could be used to induce an active immune response in human cancer patients. Such a response includes the formation of antibodies capable of activating human complement and mediating ADCC and by such mechanisms cause tumor destruction.

Immunoassays

Described herein are serological methods for determining the presence of HER2 p185. Essentially, the processes of this invention comprise incubating or otherwise exposing the sample to be tested to monoclonal antibodies and detecting the presence of a reaction product. Those skilled in the art will recognize that there are many variations of these basic procedures. These include, for example, RIA, ELISA, precipitation, agglutination, complement fixation and immuno-fluorescence. In the currently preferred procedures, the monoclonal antibodies are appropriately labeled.

The labels that are used in making labeled versions of the antibodies include moieties that may be detected directly, such as radiolabels and fluorochromes, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. The radiolabel can be detected by any of the currently available counting procedures. The preferred isotope labels are ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^{3}H, ^{32}P and ^{35}S. The enzyme label can be detected by any of the currently utilized calorimetric, spectrophotometric, fluorospectro-photometric or gasometric techniques. The enzyme is combined with the antibody with bridging molecules such as carbodiimides, periodate, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. Examples are peroxidase, alkaline phosphatase, β-glucuronidase, β-D-glucosidase, β-D-galactosidase, urease, glucose oxidase plus peroxidase, galactose oxidase plus peroxidase and acid phosphatase. Fluorescent materials which may be used include, for example, fluorescein and its derivatives, rhodamine and its derivatives, auramine, dansyl, umbelliferone, luciferia, 2,3-dihydrophthalazinediones, horseradish peroxidase, alkaline phosphatase, lysozyme, and glucose-6-phosphate dehydrogenase. The antibodies may be tagged with such labels by known methods. For instance, coupling agents such as aldehydes, carbodiimides, dimaleimide, imidates, succinimides, bid-diazotized benzadine and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. Various labeling techniques are described in Morrison, <u>Methods in</u>

Enzymology 32b, 103 (1974), Syvanen et al., J. Biol. Chem. 284, 3762 (1973) and Bolton and Hunter, Biochem J. 133, 529 (1973).

The antibodies and labeled antibodies may be used in a variety of immunoimaging or immunoassay procedures to detect the presence of cancer in a patient or monitor the status of such cancer in a patient already diagnosed to have it. When used to monitor the status of a cancer, a quantitative immunoassay procedure must be used. If such monitoring assays are carried out periodically and the results compared, a determination may be made regarding whether the patient's tumor burden has increased or decreased. Common assay techniques that may be used include direct and indirect assays. If the sample includes cancer cells, the labeled antibody will bind to those cells. After washing the tissue or cells to remove unbound labeled antibody, the tissue sample is read for the presence of labeled immune complexes. In indirect assays the tissue or cell sample is incubated with unlabeled monoclonal antibody. The sample is then treated with a labeled antibody against the monoclonal antibody (e.g., a labeled antimurine antibody), washed, and read for the presence of ternary complexes.

For diagnostic use the antibodies will typically be distributed in kit form. These kits will typically comprise: the antibody in labeled or unlabeled form in suitable containers, reagents for the incubations for an indirect assay, and substrates or derivatizing agents depending on the nature of the label. HER2 p185 controls and instructions may also be included.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof.

EXPERIMENTAL

Amplified Expression of $p185^{HER2}$ and Tyrosine Kinase Activity

A series of NIH 3T3 cell lines expressing various levels of p185 were constructed as disclosed in Hudziak et al., Proc. Natl. Acad. Sci. (USA) 84, 7159 (1987). The parental cell line had a nontransformed, TNF-α-sensitive phenotype. The control cell line (NIH 3T3 neo/dhfr) was prepared by transfection with pCVN, an expression plasmid encoding neomycin resistance as a selectable marker, and dihydrofolate reductase (which encodes methotrexate resistance and which permits amplification of associated DNA sequences). pCVN-HER2 (which encodes, in addition, the entire 1255 amino acid p185 receptor-like tyrosine kinase under the transcriptional control of the RSV-LTR) was introduced into NIH 3T3 cells in a parallel transfection. Transfectants were selected by resistance to the aminoglycoside antibiotic G418. The pCVN-HER2 primary transfectants (HER2-3) do not have a transformed morphology and fail to grow in soft agar. Stepwise amplification of HER2 expression by selection in 200nM (HER2-3_{200}), 400 nM (HER2-3_{400}), and 800 nM (HER2-3_{800}) methotrexate, however, results in transformation as judged by morphological criteria, the ability to grow in soft agar, and the ability to form tumors in nude mice.

The amplification of expression of p185 was documented by immunoprecipitation from cells that were metabolically labeled with ^{35}S-methionine. The tyrosine kinase activity associated with p185 in these cell lines was measured by autophosphorylation in vitro. For an autoradiograph of ^{35}S-methionine labeled p185, 200 μCi of ^{35}S-methionine (Amersham; 1132 Ci/mmol) was added to 1.5 ml of methionine-free labeling medium, containing 2% dialyzed fetal bovine serum. 1.0×10^6 cells of each type were counted by Coulter counter, plated in 60 mm culture dishes (Falcon), and allowed to adhere for 12 h. Following an 8 h labeling period the cells were lysed and the HER2-encoded p185 was analyzed. For an autoradiograph of self-phosphorylated HER2-receptor tyrosine kinase, the p185 was immunoprecipitated and the pellet was resuspended in 50 μl of tyrosine kinase reaction buffer. The samples were incubated at 4°C for 20 min. The self-phosphorylated p185 from the various cell lines was then visualized by autoradiography following gel electrophoresis. The molecular weight markers used were myosin (200kD) and β-galactosidase (116kD). The results showed that expression of p185 and its associated tyrosine kinase increased in parallel during amplification. Quantitative densitometry of the _in vitro_ autophosphorylation reactions showed that the tyrosine kinase activity increased at least 5 to 6-fold between HER2-3 and HER2-3_{200} and between HER2-3_{200} and HER2-3_{400}, while only a small difference was observed between HER2-3_{400} and HER2-3_{800} (see the Tyrosine Kinase column of Table 1 below).

Relative amounts of tyrosine kinase present in each of the cell types of Table 1 were determined by taking ratios of the areas under the curves obtained by scanning autoradiograms (using an LKB2202 laser densitometer). The autoradiograms had been exposed for various times to allow for linearity in the determinations, and then normalized by comparison to the HER2 primary transfectant (HER2-3).

Resistance to TNF-α

The cell lines described above were then tested for sensitivity to TNF-α and macrophage-induced cytotoxicity.

In Figure 1a, TNF-α resistance of the control cells and the HER2-transfected NIH 3T3 cells is shown. Cells were seeded into 96-well microtiter plates at a density of 5,000 cells/well in DMEM supplemented with 10% calf serum, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were allowed to adhere for 4 hrs before the addition of a range of concentrations of TNF-α. Specific activity of the TNF-α (recombinant human TNF-α) was 5 x 10^7 U/mg as determined in an L-M cell cytotoxicity assay in the presence of actinomycin D. After incubation at 37°C for 72 hr, the monolayers were washed with PBS and stained with crystal violet dye for determination of relative cell viability. These measurements were repeated six times. Results from a representative experiment are shown in Figure 1a.

In Figure 1b, macrophage-mediated cytotoxicity assays are shown. TNF-α resistant cells (neo/dhfr HTR) were derived by subculturing a clone of NIH 3T3 neo/dhfr in media containing 10,000 U/ml TNF-α. For macrophage cytotoxicity assays, NIH 3T3 neo/dhfr, HER2-3_{800} and neo/dhfr HTR cells were seeded into 96-well microtiter plates as in 1a above. Human macrophages were obtained as adherent cells from peripheral blood of healthy donors. Adherent cells were scraped and resuspended in media, activated for 4 hr. with 10 μg/ml E. coli-derived lipopolysaccharide (LPS; Sigma) and 100 U/ml of recombinant human interferon-gamma (rHuIFN-γ, Genentech, Inc.). The cell suspension was then centrifuged for 10 minutes at 1200 rpm and the resulting pellet was washed with media to remove the LPS and rHuIFN-γ. The macrophages were resuspended in media, counted, and then added to the target cells to obtain the desired effector to target ratios. After a 72 hr incubation at 37°C, the monolayers were washed with media and ^{51}Cr was added to each well for determination of viability by ^{51}Cr uptake.

Table 1

Correlation between HER2-associated tyrosine kinase levels and resistance to TNF-α

Cell Type	Percent Viability	Tyrosine Kinase
1. NIH 3T3 neo/dhfr	3.6 ± 0.6	*
2. NIH 3T3 neo/dhfr$_{400}$	8.3 ± 1.0	*
3. HER2-3	2.0 ± 0.4	1.0
4. HER2-3$_{200}$	27.5 ± 2.7	6.73
5. HER2-3$_{400}$	48.4 ± 1.4	32.48
6. HER2-3$_{800}$	58.7 ± 1.3	39.61
7. BT-20	1.6 ± 0.3	<0.1
8. MCF7	2.5 ± 0.3	0.26
9. MDA-MB-361	26.8 ± 6.6	10.65
10. MDA-MB-175-VII	31.2 ± 4.4	0.9
11. SK-BR-3	56.4 ± 5.5	31.0
12. MDA-MB-231	64.2 ± 9.3	<0.1

* not measured

Percent viability is given at 1.0 x 10^4 cytotoxicity units per ml of TNF-α. The breast tumor cell lines were obtained from the ATCC and maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

As shown in Fig. 1a and Table 1, stepwise amplification of HER2 receptor expression resulted in a parallel induction of resistance to TNF-α. The primary transfectants (HER2-3), which do not have a transformed phenotype, demonstrated little increased resistance. However, the transformed lines HER2-3$_{200}$, HER2-3$_{400}$ and HER2-3$_{800}$ do show a stepwise loss in sensitivity to TNF-α-mediated cytotoxicity as compared to NIH 3T3 neo/dhfr (Fig. 1a and Table 1), although the MDA-MB-175-VII cells had elevated p185 expression compared to the TNF-α sensitive BT20 and MCF7 cell lines. In correlation with the levels of p185 expression (Table 1), the difference in sensitivity of HER2-3$_{200}$ and HER2-3$_{400}$ (27.5%

vs. 48.4% viability at 1 x 10^4 U/ml TNF-α) is greater than the difference between HER2-3_{400} and HER2-3_{800} (48.4% vs. 58.7% viability, see Fig. 1a and Table 1). A similar result was obtained when NIH 3T3 neo/dhfr and HER2-3_{800} were compared for sensitivity to activated macrophages (Fig. 1b). These data suggest that amplification of the expression of HER2 induces resistance to TNF-α, and also show that this correlates with resistance to an important component of the early host defense mechanism, the activated macrophage. Amplification of the control plasmid (pCVN) in the cell line NIH 3T3 neo/dhfr$_{400}$ did not induce increased resistance to TNF-α (Table 1). This demonstrates that neither gene transfection or gene amplification, per se, has any effect on the sensitivity of cells to TNF-α.

The observation that NIH 3T3 cell lines expressing high levels of p185 were resistant to cytotoxicity induced by TNF-α or macrophages suggested that this may be one mechanism leading to tumor development. To test this possibility six breast tumor cell lines were screened for amplification of HER2 and sensitivity to TNF-α-mediated cytotoxicity. The results (Table 1) demonstrated that sensitivity to growth inhibition by TNF-α is inversely correlated with the expression of HER2-associated tyrosine kinase measured <u>in vitro</u> autophosphorylation assay for BT-20, MCF7, MDA-MB-361 and SK-BR-3. Two of the TNF-α-resistant breast tumor cell lines (MDA-MB-175-VII and MDA-MB-231), however, had no demonstrable amplified expression of HER2 as compared to the HER2-3 control (Table 1), although the MDA-MB-175-VII cells had elevated p185 expression compared to the TNF-α sensitive BT20 and MCF7 cell lines. These results are consistent with previous reports of the frequency of HER2 amplification in primary breast tumors and tumor-derived cell lines, and suggest the existence of other cellular mechanisms which may lead to TNF-α resistance.

Experiments also showed that overexpression of the EGF receptor, and cellular transformation by the src oncogene, correlates with resistance to TNF-α.

TNF-α Receptor Binding

In order to investigate whether the TNF-α receptor was altered in HER2-3_{800}, as opposed to NIH 3T3 neo/dhfr, the binding of ^{125}I-labeled TNF-α was compared between these cell lines. Figure 2 shows a TNF-α receptor binding analysis. Displacement curves show binding of ^{125}I-TNF-α to NIH 3T3 neo/dhfr and HER2-3_{800}. Competition binding assays were performed. Briefly, a suspension of 2.0 x 10^6 cells were incubated in a final volume of 0.5 ml of RPMI-1640 medium containing 10% fetal bovine serum. Binding of ^{125}I-TNF-α (0.2 x 10^6 cpm) to the cells was determined in the presence or absence of varying concentrations of unlabeled TNF-α at 4°C under saturation equilibrium conditions. Each data point represents the mean of triplicate determinations. After incubation overnight, cells were washed twice with incubation buffer and cell bound radioactivity was determined. Non-specific binding was <10% of total binding.

The results showed a 2-3 fold increase in total specific binding for HER2-3_{800} as compared to NIH 3T3 neo/dhfr (Fig. 2). In addition, the displacement curve for binding on HER2-3_{800} is also shifted toward lower affinity binding as compared to NIH 3T3 neo/dhfr (Fig. 2).

Production of Anti-HER2 Monoclonal Antibodies

Five female Balb/c mice were immunized with HER2 amplified NIH 3T3 transformed cells over a period of 22 weeks. The first four injections each had approximately 10^7 cells/mouse. They were administered intraperitoneally in half a milliliter of PBS on weeks 0, 2, 5, 7. Injections five and six were with a wheat germ agglutinin partially purified membrane preparation which had a whole protein concentration of about 700 μg/ml. A 100 μl/injection was administered to each mouse intraperitoneally on weeks 9 and 13. The last injection was also with the purified material but was administered three days prior to the date of fusion intravenously.

Bleeds from the mice were tested at various times in a radioimmunoprecipitation using whole cell lysates. The three mice with the highest antibody titers were sacrificed and spleens were fused with the mouse myeloma cell line X63-Ag8.653 using the general procedure of Mishell & Shiigi, Selected Methods in Cellular Immunology, W.H. Freeman & Co., San Francisco, p. 357-363 (1980) with the following exceptions. Cells were plated at a density of approximately 2 x 10^5 cells/well into ten 96 well microtiter plates. Hybrids were selected using hypoxanthine-azoserine rather than hypoxanthine-aminoptern-thymidine (HAT).

Hybridoma supernatants were tested for presence of antibodies specific for HER2 receptor by ELISA and radioimmunoprecipitation.

For the ELISA, 3.5 μg/ml of the HER2 receptor (purified on the wheat germ agglutinin column) in PBS was adsorbed to immulon II microtiter plates overnight at 4°C or for 2 hours at room temperature. Plates were then washed with phosphate buffered saline with .05% Tween 20 (PBS-TW20) to remove unbound antigen. Remaining binding sites were then blocked with 200 μl per well of 1% bovine serum albumin (BSA) in PBS-TW20 and incubated 1 hour at room temperature. Plates were washed as above and 100 μl of hybridoma supernatant was added to each well and incubated for 1 hour at room temperature. Plates were washed again and 100 μl per well of an appropriate dilution of goat anti-mouse immunoglobulin coupled to horseradish peroxidase was added. The plates were incubated again for 1 hour at room temperature and then washed as above. O-phenylene diamine was added as substrate, incubated for 15-20 minutes at room temperature and then the reaction was stopped with 2.5 M H_2SO_4. The absorbance of each well was then read at 492 nm.

For the radioimmunoprecipitation, first the wheat germ purified HER2 receptor preparation was autophosphorylated in the following manner: a kinase solution with the following final concentrations was made: .18 mCi/ml γP^{32}-ATP (Amersham), .4mM $MgCl_2$, .2mM $MnCl_2$, 10μM ATP, 35 μg/ml total protein concentration of partially purified HER2 all diluted in 20mM Hepes, 0.1% triton 10% glycerol buffer (HTG). This reaction was incubated for 30 minutes at room temperature. 50 μl hybridoma supernatant was then added to 50 μl of the kinase reaction and incubated 1 hour at room temperature. 50 μl of goat anti-mouse IgG precoated protein-A sepharose CL4B, at a sepharose concentration of 80 mg/ml, was added to each sample and incubated 1 hour at room temperature. The resulting immunocomplexes were then washed by centrifugation twice with HTG buffer and finally with .2% deoxycholate .2% Tween 20 in PBS, in a microfuge and aspirated between washes. Reducing sample buffer was added to each sample and samples were heated at 95°C for 2-5 minutes, insoluble material was removed by centrifugation and the reduced immunocomplex was loaded onto a 7.5% polyacrylamide gel containing SDS. The gel was run at 30 amp constant current and an autoradiograph was obtained from the finished gel.

Approximately 5% of the total well supernatants reacted with the HER2 receptor in the ELISA and/or radioimmunoprecipitation. From this initial 5% (about 100), some hybrids produced low affinity antibodies and others suffered from instability and stopped secreting antibodies leaving a total of 10 high affinity stable HER2 specific antibody producing cell lines. These were expanded and cloned by limiting dilution (Oi, V.T. and Herzenberg, L.A., "Immunoglobulin Producing Hybrid Cell Lines" in <u>Selected Methods in Cellular Immunology</u>, p. 351-372 Mishell, B.B. and Shiigi, S.M. (eds.), W.H. Freeman and Co. (1980)). Large quantities of specific monoclonal antibodies were produced by injection of cloned hybridoma cells in pristaned primed mice to produce ascitic tumors. Ascites were then collected and purified over a protein-A sepharose column.

Screening of Antibodies

The 10 high affinity monoclonal antibodies were then screened in a number of assays for anti-transformation or anti-tumor cell activity. Monoclonal antibodies were selected on the basis of growth inhibiting activity against the human tumor line SK BR3 which is derived from a breast tumor and contains an amplified HER2 gene and overexpresses the HER2 p185 tyrosine kinase. The initial screen used conditioned medium (medium in which the cells were grown for several days containing any secreted products of the cells including antibodies produced by the cells) from the hybridoma cell lines.

SK BR3 cells were plated at 20,000 cells/35 mm dish. Either conditioned medium from the hybridoma parent line (producing everything but anti-HER2 monoclonals) as a control, or the anti-HER2 monoclonals were added. After 6 days, the total number of SK BR3 cells were counted using an electronic Coulter cell counter. Cells were grown in a 1:1 mixture of F-12 and DMEM supplemented with 10% fetal bovine serum, glutamine, and penicillinstreptomycin. The volume per plate was 2 mls/35mm dish. 0.2 mls of myeloma conditioned medium was added per 35mm dish. Each control or anti-HER2 MAb was assayed in duplicate and the two counts averaged.

The result of the survey is shown in Figure 3. Monoclonal antibody 4D5 markedly inhibited the growth of the breast tumor line SK BR3. Other anti-HER2 antibodies inhibited growth to a significant but lesser extent (egs., MAbs 3E8 and 3H4). Still other anti-HER2 antibodies (not shown) did not inhibit growth.

A repeat experiment using purified antibody rather than hybridoma conditioned medium confirmed the results of Figure 3. Figure 4 is a dose response curve comparing the effect of an irrelevant monoclonal antibody (anti-HBV) and monoclonal antibody 4D5 (anti-HER2) on the growth of the SK BR3 cell line in 10% fetal bovine serum.

Down Regulation of the HER2 Receptor

The SK BR3 cells were pulse labeled with 100uci ^{35}S-methionine for 12 hours in methionine-free medium. Then either an irrelevant (anti-Hepatitis B surface antigen) or anti-HER2 MAb (4D5) was added to the cells at 5 μg/ml. After 11 hours, the cells were lysed and HER2 p185 immunoprecipitates and the proteins were analyzed by a 7.5% acrylamide gel followed by autoradiography. The SDS-PAGE gel of the ^{35}S-methionine labeled HER2 p185 from SK BR3 cells demonstrated that the HER2 levels are downregulated by MAb 4D5.

Treatment of Breast Tumor Cells with Monoclonal Antibodies and TNF-α

SK-BR-3 breast tumor cells were seeded at a density of 4×10^4 cells per well in 96-well microtiter plates and allowed to adhere for 2 hours. The cells were then treated with different concentrations of anti-HER2 monoclonal antibody (MAb) 4D5 or irrelevant isotype matched (anti-rHuIFN-γ MAb) at 0.05, 0.5 or 5.0 μg/ml for 4 hours prior to the addition of 100, 1,000 or 10,000 U/ml rHuTNF-α. After a 72 hour incubation, the cell monolayers were stained with crystal violet dye for determination of relative percent viability (RPV) compared to control (untreated) cells. Each treatment group consisted of 6 replicates. The results are shown in Figures 5 and 6. These Figures show that incubation of cells overexpressing HER2 receptor with antibodies directed to the extracellular domain of the receptor induce sensitivity to the cytotoxic effects of TNF-α. Equivalent treatment of breast tumor cells MDA-MB-175 VII gave similar results (see Figure 7). Treatment of human fetal lung fibroblasts (WI-38) with MAb resulted in no growth inhibition or induction of sensitivity to TNF-α as expected.

Treatment of NIH 3T3 Cells Overexpressing HER2 p185 with Monoclonal Antibodies and TNF-α

NIH 3T3 HER2-3_{400} cells were treated with different concentrations of anti-HER2 MAbs as in the above described treatment of SK-BR3 cells. The results for MAb 4D5 are shown in Figure 8. The results indicate that cells other than of breast tumor cell lines which overexpress the HER2 receptor are growth inhibited by antibodies to the HER2 receptor, and sensitivity to TNF-α is induced in the presence of these antibodies.

In Vivo Treatment of NIH 3T3 Cells Overexpressing HER2 with Anti-HER2 IgG2A Monoclonal Antibodies

NIH 3T3 cells transfected with either a HER2 expression plasmid (NIH $3T3_{400}$) or the neo-DHFR vector were injected into nu/nu (athymic) mice subcutaneously at a dose of 10^6 cells in 0.1 ml of phosphate-buffered saline. On days 0, 1, 5 and every 4 days thereafter, 100 μg (0.1 ml in PBS) of either an irrelevant or anti-HER2 monoclonal antibody of the IgG2A subclass was injected intraperitoneally. Tumor occurrence and size were monitored for the 1 month period of treatment.

Table 2

Group #	Cell Line	Treatment	# Tumors/ # Animals	Tumor Size of Survivors: Length x Width Average in mm^2 at 31 Days
1	HER2 ($3T3_{400}$)	Irrelevant MAb (anti-Hepatitis B Virus)	6/6	401
2	HER2 ($3T3_{400}$)	2H11 anti-HER2	2/6	139
3	HER2 ($3T3_{400}$)	3E8 anti-HER2	0/6	0
4	neo/DHFR	None	0/6	0

Table 2 shows that the 2H11 MAb has some anti-tumor activity (MAb 2H11 has very slight growth inhibiting properties when screened against tumor line SK BR3) and the 3E8 MAb gives 100% tumor growth inhibition during the course of the experiment.

While the invention has been described in what is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such modifications and equivalents.

What is claimed is:

1. A monoclonal antibody specifically binding the extracellular domain of the HER2 receptor.

2. A monoclonal antibody as in claim 1 which is capable of inhibiting the HER2 receptor function.

3. A monoclonal antibody as in claim 1 which is capable of inhibiting serum activation of HER2 receptor function.

4. A monoclonal antibody as in claim 1 which is a murine monoclonal antibody.

5. A monoclonal antibody as in claim 1 which is a murine-human hybrid antibody.

6. A monoclonal antibody as in claim 1 wherein said antibody specifically blocks the ligand binding site of the HER2 receptor.

7. A monoclonal antibody as in claim 1 which down regulates the HER2 receptor.

8. A monoclonal antibody as in claim 1 wherein said antibody is capable of activating complement.

9. A monoclonal antibody as in claim 1, wherein said antibody is capable of mediating antibody dependent cellular cytotoxicity.

10. An immunotoxin which is a conjugate of a cytotoxic moiety and the antibody of claim 1.

11. A hybridoma producing the monoclonal antibody of claim 1.

12. An assay for detecting a tumor comprising the steps of:

exposing cells to antibodies specifically binding the extracellular domain of the HER2 receptor, and

determining the extent of binding of said antibodies to said cells.

13. An assay as in claim 12 wherein said antibodies are monoclonal antibodies.

14. An assay as in claim 12 wherein the assay is an ELISA assay.

15. An assay as in claim 12 wherein said cells remain within the body of a mammal, said antibodies are tagged with a radioactive isotope and administered to the mammal, and the extent of binding of said antibodies to said cells is observed by external scanning for radioactivity.

16. A method of inhibiting the growth of tumor cells comprising:

administering to a patient a therapeutically effective amount of antibodies capable of inhibiting the HER2 receptor function.

17. A method as in claim 16 wherein said antibodies specifically blocks the ligand binding site of the HER2 receptor.

18. A method as in claim 16 wherein said antibodies are conjugated to a cytotoxic moiety.

19. A method as in claim 16 wherein said antibodies are capable of activating complement.

20. A method as in claim 16 wherein said antibodies are capable of mediating antibody dependent cellular cytotoxicity.