21. A method as in claim 16 wherein said antibodies are monoclonal antibodies.

22. A method as in claim 16 wherein the tumor cells comprise a carcinoma selected from human breast, renal, gastric and salivary gland carcinomas, or other tumor cell types expressing the HER2 receptor.

23. A method of treating tumor cells comprising the steps of:

administering to a patient a therapeutically effective amount of antibodies capable of inhibiting growth factor receptor function; and

administering to a patient a therapeutically effective amount of a cytotoxic factor.

24. A method as in claim 23 wherein said cytotoxic factor is selected from the group consisting of TNF-α, TNF-β, IL-1, IFN-γ and IL-2.

25. A method as in claim 23 wherein said cytotoxic factor is TNF-α

26. A method as in claim 23 wherein said antibodies interrupt an autocrine growth cycle.

27. A method as in claim 23 wherein said antibodies specifically bind a growth factor receptor.

28. A method as in claim 27 wherein the growth factor receptor is selected from the group consisting of the EGF receptor and the HER2 receptor.

29. A method as in claim 23 wherein said antibodies specifically bind a growth factor.

30. A method as in claim 29 wherein said growth factor is selected from the group consisting of EGF, TGF-α and TGF-β.

31. A method as in claim 23 wherein said antibodies are monoclonal antibodies.

32. A method as in claim 23 wherein said antibodies are conjugated to a cytotoxic moiety.

33. A method as in claim 23 wherein said antibodies are capable of activating complement.

34. A method as in claim 23 wherein said antibodies are capable of mediating antibody dependent cellular cytotoxicity.

35. A method as in claim 23 wherein the tumor cells comprise a carcinoma selected from human breast, renal, gastric and salivary gland carcinomas.

36. An assay for receptors and other proteins having increased tyrosine kinase activity comprising the steps of:
(a) exposing cells suspected to be TNF-α sensitive to TNF-α;
(b) isolating those cells which are TNF-α resistant;
(c) screening the isolated cells for increased tyrosine kinase activity; and
(d) isolating receptors and other proteins having increased tyrosine kinase activity.

37. A composition suitable for administration to a patient having a growth factor receptor dependent tumor comprising (a) antibodies capable of inhibiting growth factor receptor function, and (b) a cytotoxic factor.

38. A composition as in claim 37 wherein the cytotoxic factor is selected from the group consisting of TNF-α, TNF-β, IL-1, IFN-γ and IL-2.

39. An immunotoxin as in Claim 10 wherein the cytotoxic moiety is ricin A chain.

Fig.1a

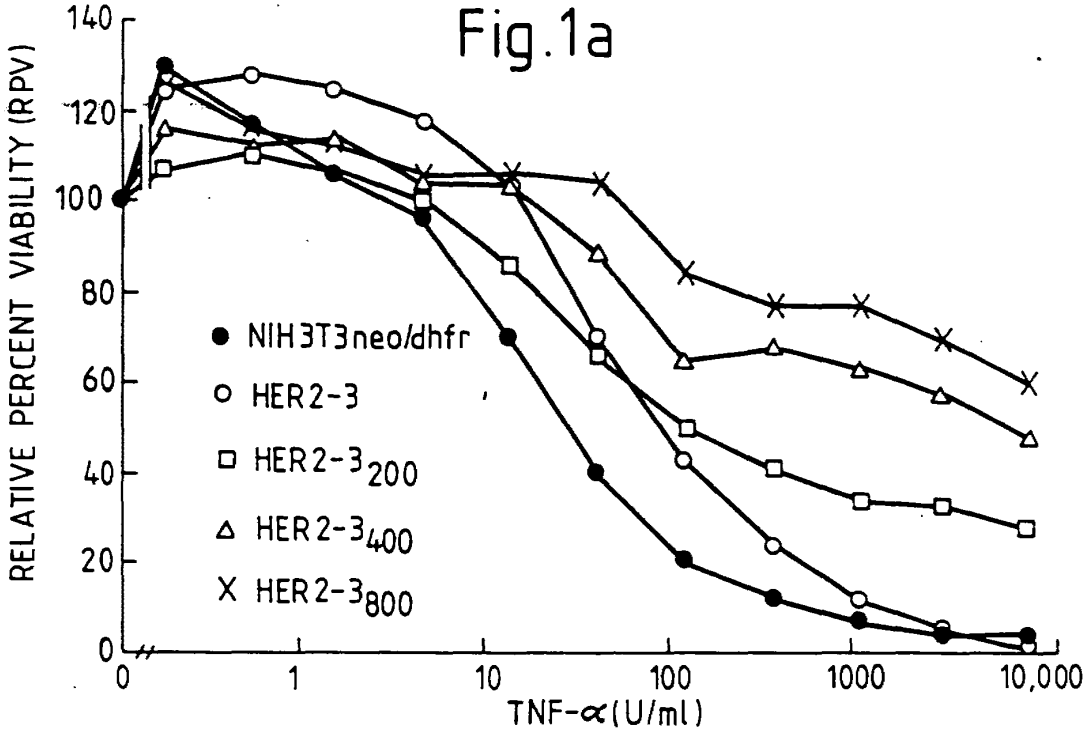

RELATIVE PERCENT VIABILITY (RPV)
140
120
100
80
60
40
20
0
NIH3T3neo/dhfr
HER2-3
HER2-3200
HER2-3400
HER2-3800
0
1
10
100
1000
10,000
TNF-α(U/ml)

Fig.1b

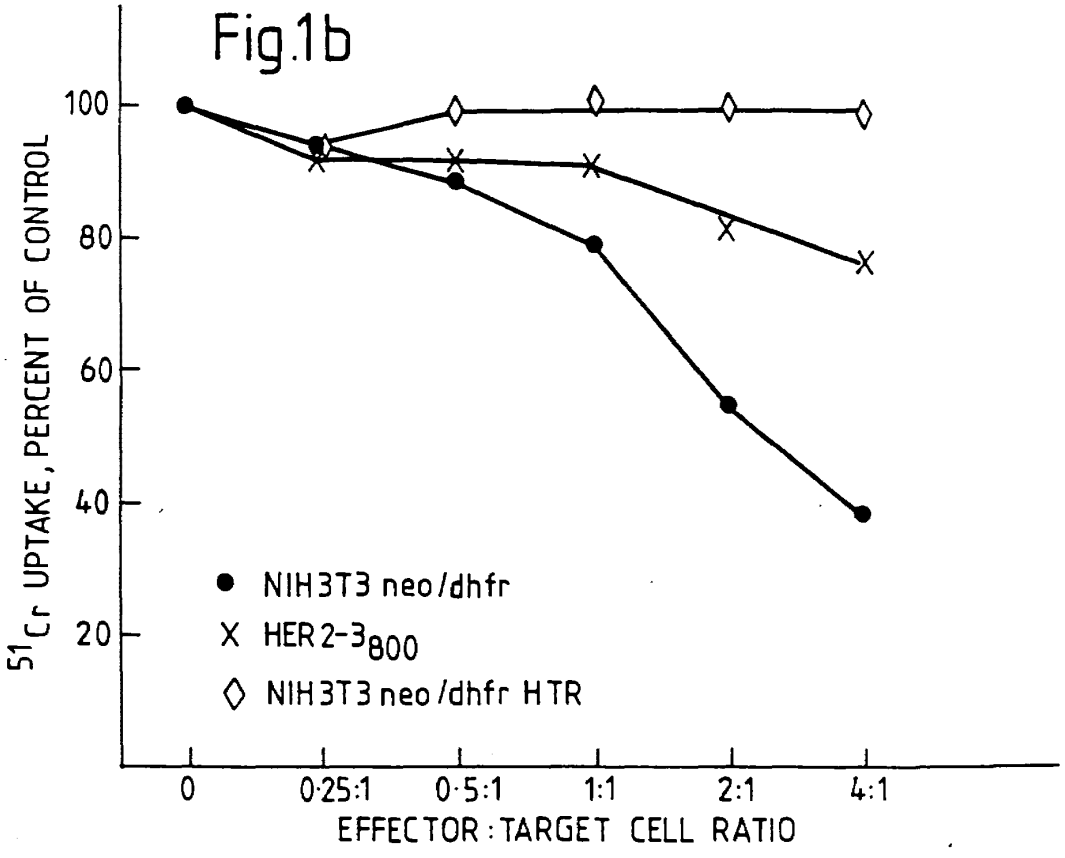

51Cr UPTAKE, PERCENT OF CONTROL
100
80
60
40
20
NIH3T3 neo/dhfr
HER2-3800
NIH3T3 neo/dhfr HTR
0
0·25:1
0·5:1
1:1
2:1
4:1
EFFECTOR:TARGET CELL RATIO

Fig. 2

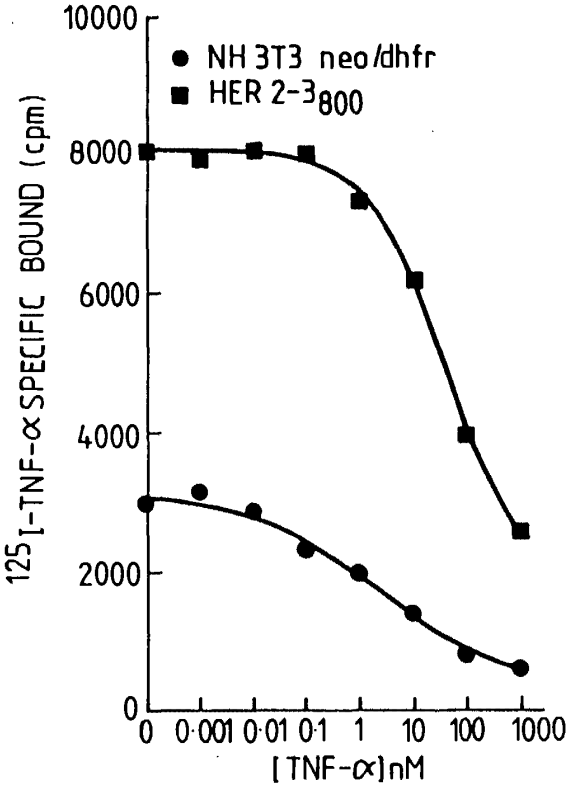

10000
8000
6000
4000
2000
0
● NH 3T3 neo/dhfr
■ HER 2-3800
125I-TNF-α SPECIFIC BOUND (cpm)
0 0·001 0·01 0·1 1 10 100 1000
[TNF-α]nM

Fig. 3

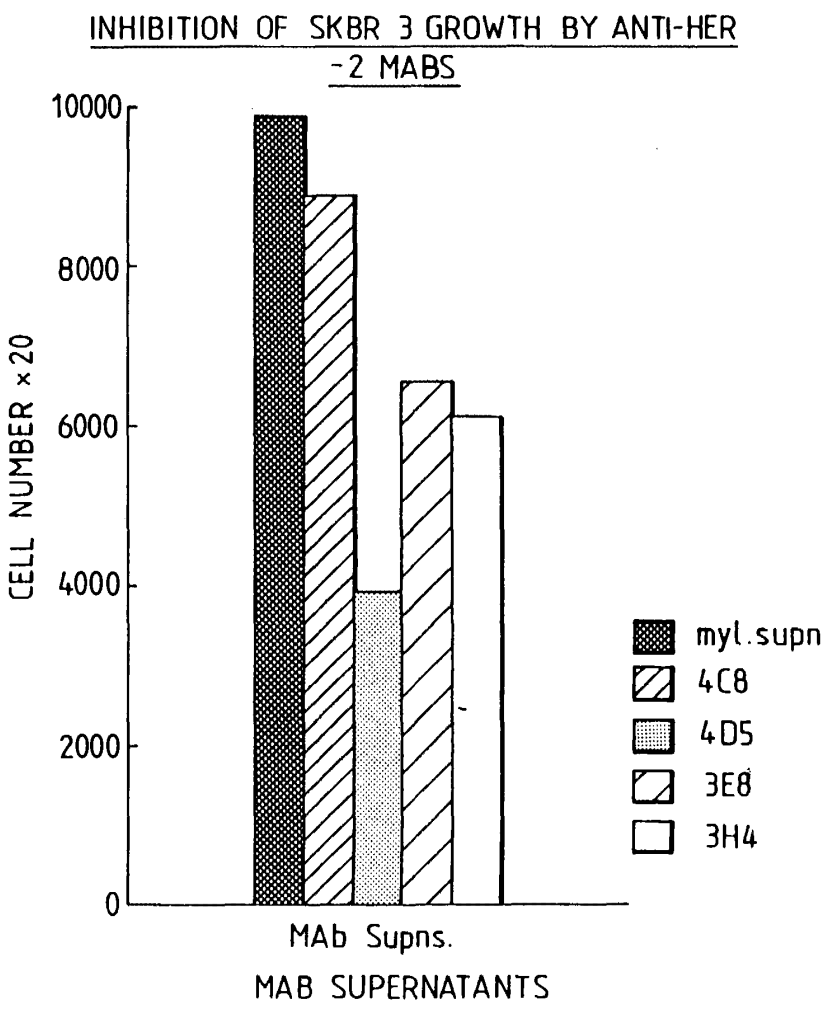

INHIBITION OF SKBR 3 GROWTH BY ANTI-HER -2 MABS
10000
8000
6000
4000
2000
0
CELL NUMBER x 20
myl.supn
4C8
4D5
3E8
3H4
MAb Supns.
MAB SUPERNATANTS

Fig. 4

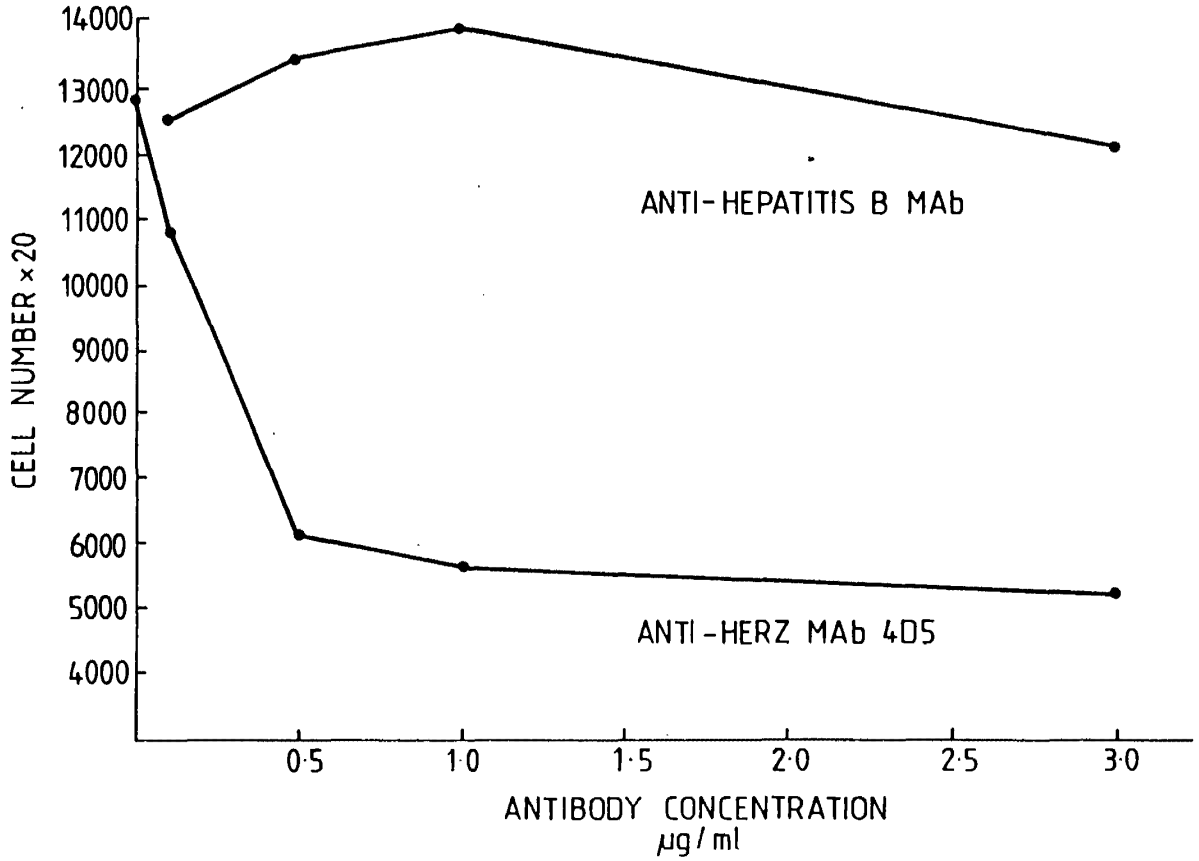

14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
CELL NUMBER × 20
ANTI-HEPATITIS B MAb
ANTI-HERZ MAb 4D5
0·5
1·0
1·5
2·0
2·5
3·0
ANTIBODY CONCENTRATION
µg/ml

Fig. 6a

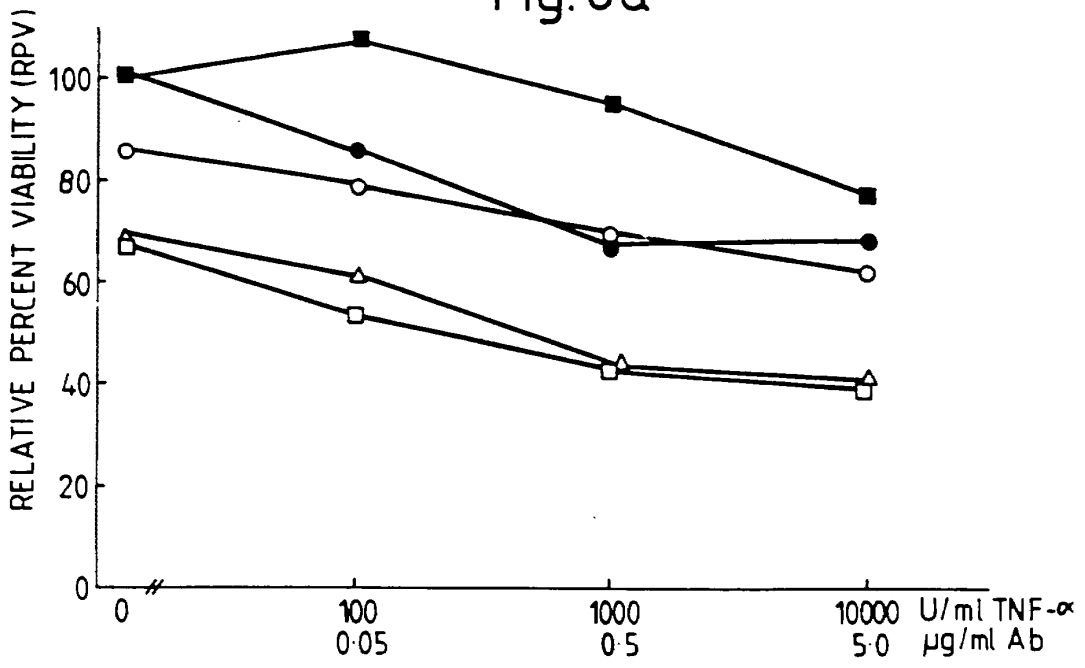

RELATIVE PERCENT VIABILITY (RPV)
100
80
60
40
20
0
0
100
0·05
1000
0·5
10000
5·0
U/ml TNF-α
μg/ml Ab

Fig. 6b

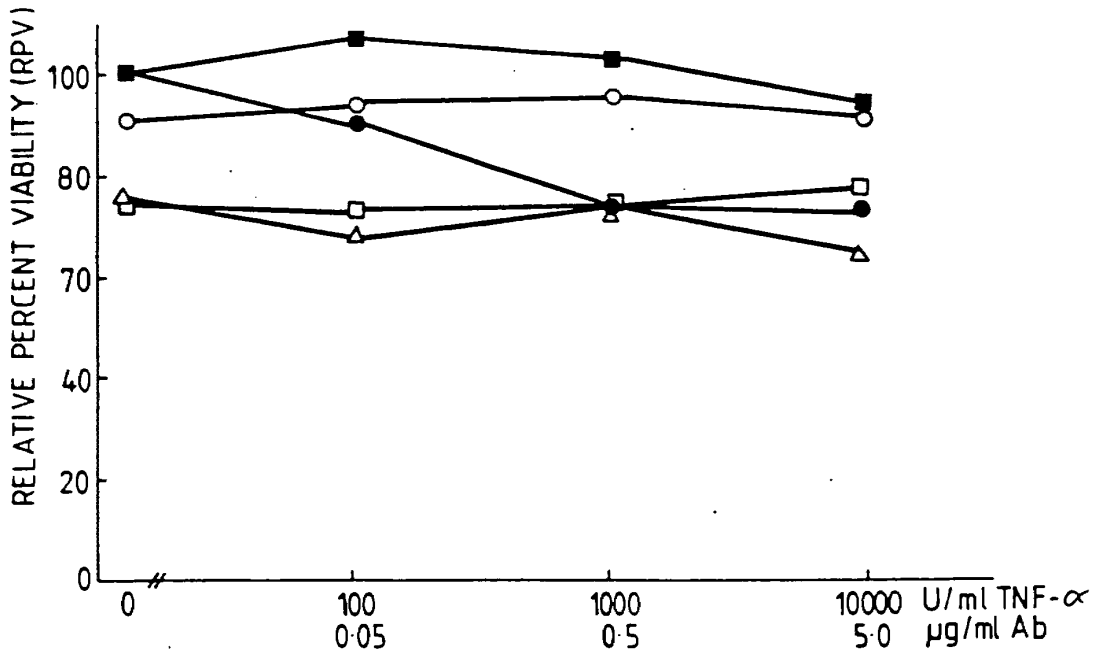

RELATIVE PERCENT VIABILITY (RPV)
100
80
70
40
20
0
0
100
0·05
1000
0·5
10000
5·0
U/ml TNF-α
μg/ml Ab

Fig.5a

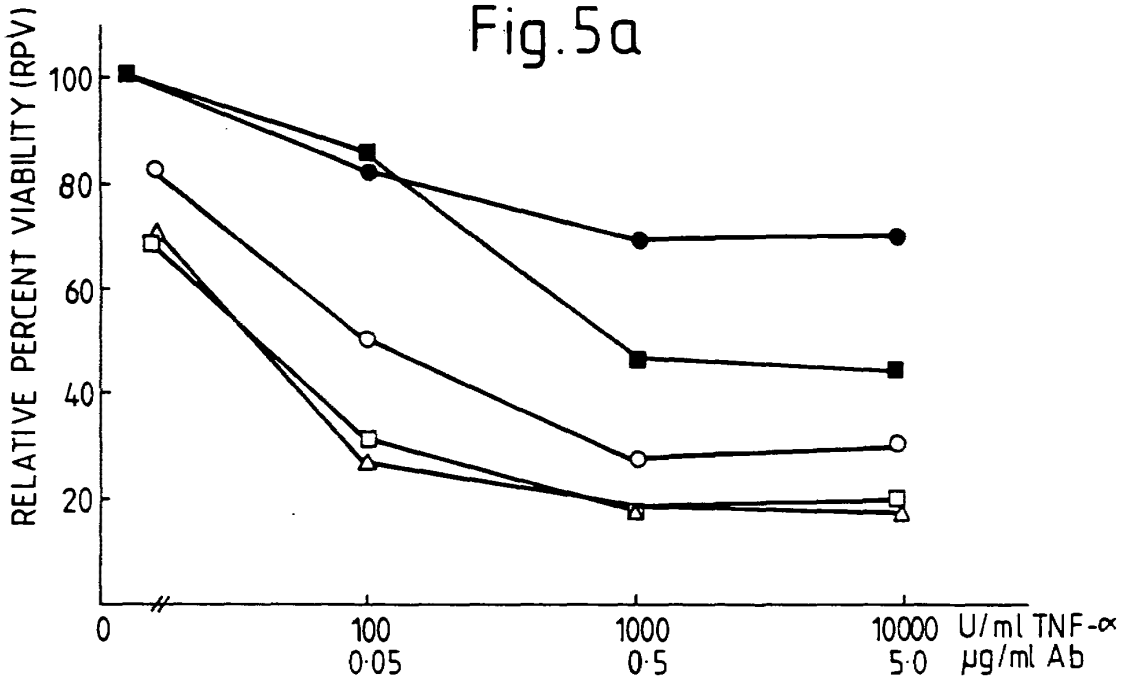

RELATIVE PERCENT VIABILITY (RPV)
100
80
60
40
20
0
100
0·05
1000
0·5
10000
5·0
U/ml TNF-∝
µg/ml Ab

Fig.5b

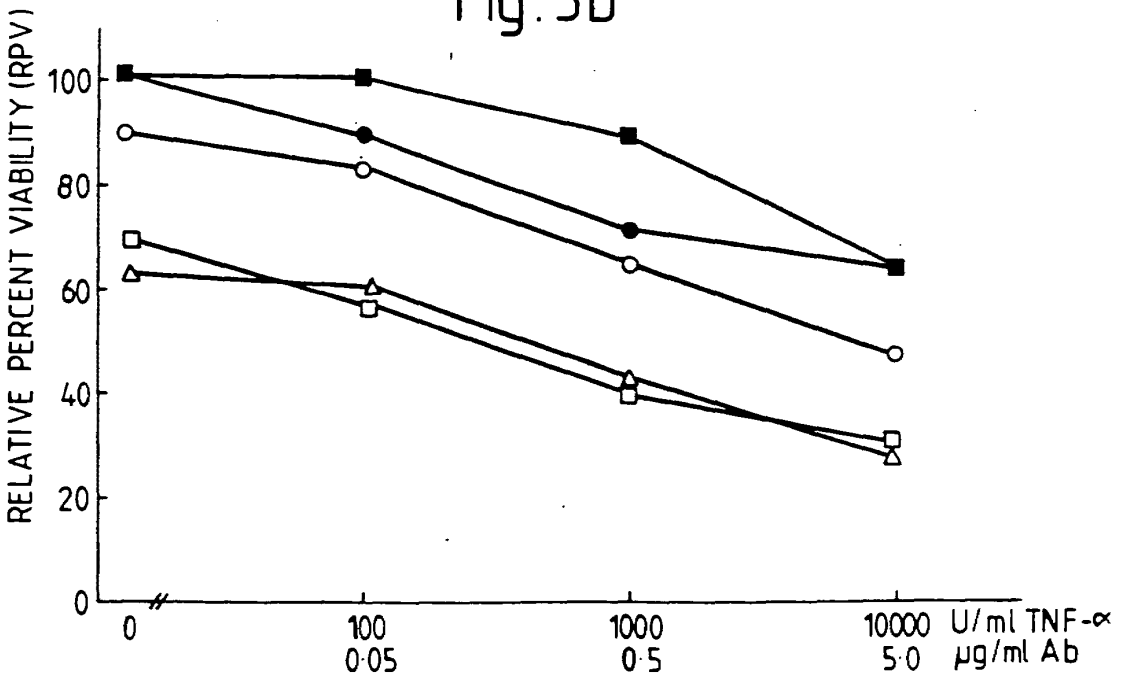

RELATIVE PERCENT VIABILITY (RPV)
100
80
60
40
20
0
0
100
0·05
1000
0·5
10000
5·0
U/ml TNF-∝
µg/ml Ab

Fig.7

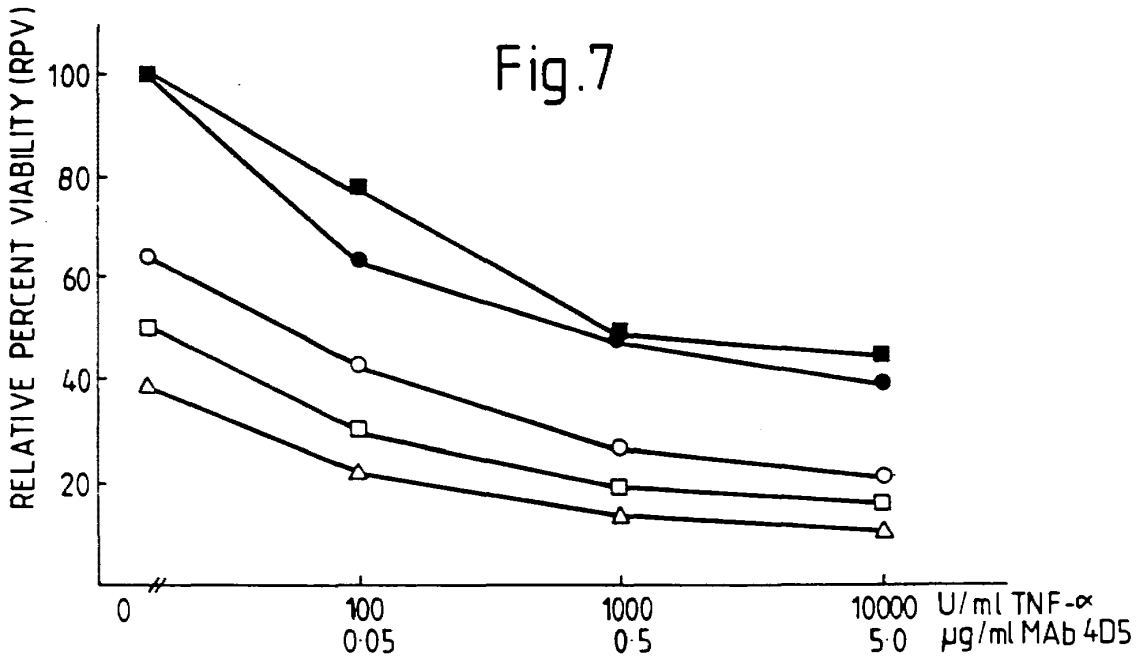
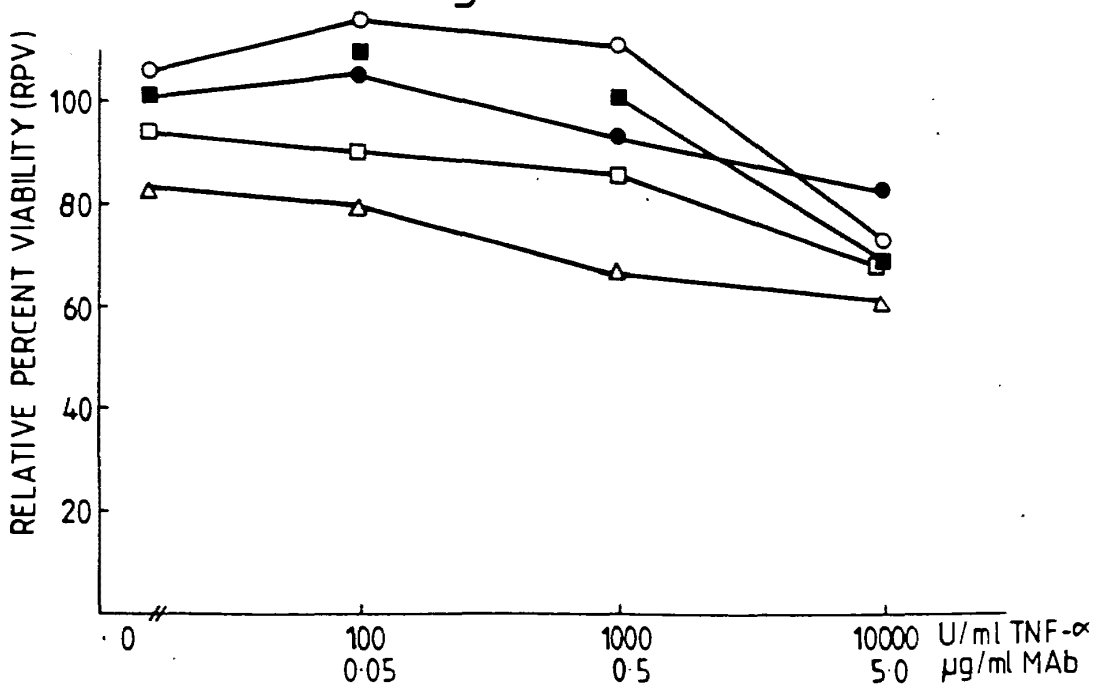

RELATIVE PERCENT VIABILITY (RPV)
100
80
60
40
20
0
100
0·05
1000
0·5
10000
5·0
U/ml TNF-α
μg/ml MAb 4D5

Fig.8


RELATIVE PERCENT VIABILITY (RPV)
100
80
60
40
20
0
100
0·05
1000
0·5
10000
5·0
U/ml TNF-α
μg/ml MAb

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/00051

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

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

IPC[4]: C 12 P 21/00; C 12 N 15/00; C 12 N 5/00; G 01 N 33/574; A 61 K 39/395

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	A 61 K; C 12 N

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

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	WO, A, 85/03357 (ICRF PATENTS LTD; YEDA RESEARCH & DEVELOPMENT CO. LTD; GENENTECH INC.) 1 August 1985 see page 36, line 15 - page 40, line 3 --	12-15
Y	J. Natl. Cancer Instit., vol. 79, no. 3, September 1987, R.E. Sobol et al.: "Epidermal growth factor receptor expression in human lung carcinomas defined by a monoclonal antibody", pages 403-407 see page 403, abstract --	12-15
Y	Cancer Res., vol. 47, no. 14, July 1987, U. Rodeck et al.: "Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: immunologically mediated and effector cell-independent effects", pages 3692-3696 ./.	1-9

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2nd May 1989	16.06.89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER POTTEN

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

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

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	see page 3695	
	--	
Y	Cancer Res., vol. 46, no. 11, November 1986, H. Masui et al.: "Mechanism of anti-tumor activity in mice for anti-epidermal growth factor receptor monoclonal antibodies with different isotypes", pages 5592-5598 see page 5592, abstract	1-9
	--	
Y	Proc. Natl. Acad. Sci., USA, vol. 83, December 1986, J.A. Drebin et al.: "Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen", pages 9129-9133 see page 9129, abstract cited in the application	1-9
	--	
Y	Journal of Cellular Biochemistry, Growth Regulation of Cancer, vol. 35, no. 4, 1987, Alan R. Liss, Inc., U. Rodeck et al.: "Interactions between growth factor receptors and corresponding monoclonal antibodies in human tumors", pages 315:JCB-320:JCB, 65:GRC-70:GRC see page 65:GRC	1-9
	--	
Y	Cell, vol. 41, July 1985, MIT, J.A. Drebin et al.: "Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies", pages 695-706 see page 695, abstract cited in the application	1-9

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

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

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

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

* 16-35, see PCT-rule 39.1(IV); methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

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

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

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

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

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

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

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

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8900051 SA 26543

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 09/06/89
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- 8503357	01-08-85	AU-A- 3934085	09-08-85
		EP-A- 0171407	19-02-86
		JP-T- 61501168	12-06-86

EPO FORM P079

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

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER 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

(71) Applicant: PROTEIN DESIGN LABS, INC. [US/US]; 3181 Porter Drive, Palo Alto, CA 94304 (US).

(72) Inventors: QUEEN, Cary, L.; 1300 Oak Creek Drive, Palo Alto, CA 94304 (US). SELICK, Harold, Edwin; 1673 Sunnyslope Avenue, Belmont, CA 94002 (US).

(74) Agent: SMITH, William, M.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).

(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.

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, 315, 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 239: 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 321:731-734 (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, Serr , and various Pseudomonas species. In these prokaryoti sts, one can also make expression vectors, which wi 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. unol. Rev. 89:49-68 (1986), which is incorporated he y reference), and necessary processing information si , such as ribosome binding sites, RNA splice sites, polyadenylation site , and transcriptional terminator sequences. Preferred exp ion control sequences are promoters derived from SV40 with hancer (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, kidneyr, 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 cofactors, enzyme inhibitors, ligands (particularly haptens), etc. Numerous types of immunoassays are available and are well known to those skilled in the art.

Kits can also be supplied for use with the subject antibodies in the protection against or detection of a cellular activity or for the presence of a selected antigen. Thus, the subject antibody composition of the present invention may be provided, usually in a lyophilized form in a container, either alone or in conjunction with additional antibodies specific for the desired cell type. The antibodies, which may be conjugated to a label or toxin, or unconjugated, are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of active antibody, and usually present in total amount of at least about 0.001% wt. based again on the antibody concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1 to 99% wt. of the total composition. Where a second antibody capable of binding to the chimeric antibody is employed in an assay, this will usually be present in a separate vial. The second antibody is typically conjugated to a label and formulated in an analogous manner with the antibody formulations described above.

The following examples are offered by way of illustration, not by limitation.

EXPERIMENTAL

Design of genes for human-like light and heavy chains

The sequence of the human antibody Eu (Sequences of Proteins of Immunological Interest, Kabat, E., et al., U.S. Dept. of Health and Human Services, 1983) was used to provide the framework of the humanized antibody, because the amino acid sequence of the heavy chain of anti-Tac is more homologous to the heavy chain of this antibody than to any other heavy chain sequence in the National Biomedical Foundation Protein Identification Resource.

To select the sequence of the humanized heavy chain, the anti-Tac heavy chain sequence (see, commonly assigned U.S.S.N.'s 186,862 and 223,037, which are incorporated herein by reference) was aligned with the sequence of the Eu heavy chain (Figure 1). At each position, the Eu amino acid was selected for the humanized sequence, unless that position fell in any one of the following categories, in which case the anti-Tac amino acid was selected.

(1) The position fell within a complementarity determining region (CDR), as defined by Kabat, et al., op. cit. (amino acids 31-35, 50-66, 99-106);

(2) The Eu amino acid was unusual for human heavy chains at that position, whereas the anti-Tac amino acid was typical for human heavy chains at that position (amino acids 27, 93, 95, 98, 107-109, 111);

(3) The position was immediately adjacent to a CDR in the amino acid sequence of the anti-Tac heavy chain (amino acids 30 and 67).

(4) 3-dimensional modeling of the anti-Tac antibody suggested that the amino acid was physically close to the antigen binding region (amino acids 48 and 68).

Some amino acids fell in more than one of these categories but are only listed in one.

To select the sequence of the humanized light chain, the anti-Tac light chain sequence was aligned with the sequence of the Eu light chain (Figure 2). The Eu amino acid was selected at each position, unless the position again fell into one of the categories (1) - (4), (with light chain replacing heavy chain in the category definitions):

(1) CDRs (amino acids 24-34, 50-56, 89-97).

(2) Anti-Tac amino acid more typical than Eu (amino acids 48 and 63).

(3) Adjacent to CDRs (no amino acids; Eu and anti-Tac were already the same at all these positions).

(4) Possible 3-dimensional proximity to binding region (amino acid 60).

The actual nucleotide sequence of the heavy (Figure 3) and light chain (Figure 4) genes were selected as follows:

(1) the nucleotide sequences code for the amino acid sequences chosen as described above.

(2) 5' of these coding sequences, the nucleotide sequences code for a leader (signal) sequence, namely the leader of the light chain of the antibody MOPC 63 and the leader of the heavy chain of the antibody PCH 108A (Kabat et al., op. cit.). These leader sequences were chosen as typical of antibodies.

(3) 3' of the coding sequences, the nucleotide sequences are the sequences that follow the mouse light chain J5 segment and the mouse heavy chain J2 segment, which are part of the anti-Tac sequences. These sequences are included because they contain splice donor signals.

(4) At each end of the sequence is an Xba I site to allow cutting at the Xba I sites and cloning into the Xba I site of a vector.

Construction of humanized light and heavy chain genes

To synthesize the heavy chain, four oligonucleotides HES12, HES13, HES14, HES15 (Figure 5A) were synthesized using an Applied Biosystems 380B DNA synthesizer. Two of the oligonucleotides are part of each strand of the heavy chain, and each oligonucleotide overlaps the next one by about 20 nucleotides to allow annealing (Figure 5B). Together, the oligonucleotides cover the entire humanized heavy chain (Figure 3) with a few extra nucleotides at each end to allow cutting at the Xba I sites. The oligonucleotides were purified from polyacrylamide gels.

Each oligonucleotide was phosphorylated using ATP and T4 polynucleotide kinase by standard procedures (see, Maniatis, op. cit.). To anneal the phosphorylated oligonucleotides, they were suspended together in 40 ul of TA (33 mM Tris acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate) at a concentration of about 3.75 uM each, heated to 95 deg for 4 min. and cooled slowly to 4 deg. To synthesize the complete gene from the oligonucleotides by synthesizing the opposite strand of each oligonucleotide (Figure 5B), the following components were added in a final volume of 100ul:

10 ul	annealed oligonucleotides
0.16 mM each	deoxyribonucleotide
0.5 mM	ATP
0.5 mM	DTT
100 ug/ml	BSA
3.5 ug/ml	T4 g43 protein (DNA polymerase)
25 ug/ml	T4 g44/62 protein (polymerase accessory protein)
25 ug/ml	45 protein (polymerase accessory protein)

The mixture was incubated at 37 deg for 30 min. Then 10 u of T4 DNA ligase was added and incubation at 37 deg resumed for 30 min. The polymerase and ligase were inactivated by incubation of the reaction at 70 deg for

15 min. To digest the gene with Xba I, to the reaction was added 50 ul of 2x TA containing BSA at 200 ug/ml and DTT at 1 mM, 43 ul of water, and 50 u of Xba I in 5 ul. The reaction was incubated for 3 hr at 37 deg, and run on a gel. The 431 bp Xba I fragment was purified from a gel and cloned into the Xba I site of the plasmid pUC19 by standard methods. Four plasmid isolates were purified and sequenced using the dideoxy method. One of these had the correct sequence (Figure 3).

To synthesize the light chain, four oligonucleotides JFD1, JFD2, JFD3, JFD4 (Figure 6A) were synthesized. Two of the oligonucleotides are part of each strand of the light chain, and each oligonucleotide overlaps the next one by about 20 nucleotides to allow annealing (Figure 6B). Together, the oligonucleotides cover the entire humanized light chain (Figure 4) with a few extra nucleotides at each end to allow cutting at the Xba I sites. The oligonuclootides were purified from polyacrylamide gels.

The light chain gene was synthesized from these olignucleotides in two parts. 0.5 ug each of JFD1 and JFD2 were combined in 20 ul sequenase buffer (40 mM Tris-HCl, pH 7.5, 20 mM magnesium chloride, 50 mM sodium chloride), heated at 70 deg for 3 min and allowed to cool slowly to 23 deg in order for the oligonucleotides to anneal. JFD3 and JFD4 were treated in the same way. Each reaction was made 10 mM in DTT and 0.5 mM in each deoxyribonucleotide and 6.5 u of sequenase (US Biochemicals) was added, in a final volume of 24 ul, and incubated for 1 hr at 37 deg to synthesize the opposite strands of the oligonucleotides. Xba I and Hind III were added to each reaction to digest the DNA (there is a Hind III site in the region where JFD2 and JFD3 overlap and therefore in each of the synthesized DNAs; Figure 6B). The reactions were run on polyacrylamide gels, and the Xba I - Hind III fragments were purified and cloned into pUC18 by standard methods. Several plasmid isolates for each fragment were sequenced by the dideoxy method, and correct ones chosen.

Construction of plasmids to express humanized light and heavy chains

The heavy chain Xba I fragment was isolated from the pUC19 plasmid in which it had been inserted and then inserted into the Xba I site of the vector pVγ1 (see, commonly assigned U.S.S.N. 223,037) in the correct orientation by standard methods, to produce the plasmid pHuGTAC1 (Figure 7). This plasmid will express high levels of a complete heavy chain when transfected into an appropriate host cell.

The two light chain Xba I - Hind III fragments were isolated from the pUC18 plasmids in which they had been inserted. The vector plasmid pVκ1 (see, commonly assigned U.S.S.N. 223,037) was cut with Xba I, dephosphorylated and ligated with the two fragments by standard methods. The desired reaction product has the circular form: vector - Xba I - fragment 1 - Hind III - fragment 2 - Xba I - vector. Several plasmid isolates were analyzed by restriction mapping and sequencing, and one with this form chosen. This plasmid, pHuLTAC (Figure 8), therefore contains the complete humanized light chain (Figure 4) and will express high levels of the light chain when transfected into an appropriate host cell.

Synthesis and affinity of humanized antibody

The plasmids pHuGTAC1 and pHuLTAC were transfected into mouse Sp2/0 cells, and cells that integrated the plasmids were selected on the basis of resistance to mycophenolic acid and/or hygromycin B conferred by the gpt and hyg genes on the plasmids (Figures 7,8) by standard methods. To verify that these cells secreted antibody that binds to the IL-2 receptor, supernatant from the cells was incubated with HUT-102 cells that are known to express the IL-2 receptor. After washing, the cells were incubated with fluorescein-conjugated goat anti-human antibody, washed, and analyzed for fluorescence on a FACSCAN cytofluorometer. The results (Figure 9A), clearly show that the humanized antibody binds to these cells, but not to Jurkat T-cells that do not express the IL-2 receptor (Figure 9D). As controls, the

original mouse anti-Tac antibody was also used to stain these cells (Figure 9B,C), giving similar results.

For further experiments, cells producing the humanized antibody were injected into mice, and the resultant ascites collected. Humanized antibody was purified to substantial homogeneity from the ascites by passage through an affinity column of goat anti-human immunoglobulin antibody, prepared on an Affigel-10 support (Bio-Rad Laboratories, Inc., Richmond, CA) according to standard techniques. To determine the affinity of the humanized antibody relative to the original anti-Tac antibody, a competitive binding experiment was performed. About 5×10^5 HUT-102 cells were incubated with known quantities (10 - 40 ng) of the anti-Tac antibody and the humanized anti-Tac antibody for 10 min at 4 deg. Then 100 ng of biotinylated anti-Tac was added to the cells and incubated for 30 min at 4 deg. This quantity of anti-Tac had previously been determined to be sufficient to saturate the binding sites on the cells, but not to be in large excess. Then the cells were washed twice with 2 ml of phosphate buffered saline (PBS) containing 0.1% sodium azide. The cells were then incubated for 30 min at 4 deg with 250 ng of phycoerythrin-conjugated avidin, which bound to the biotinylated anti-Tac already bound to the cells. The cells were washed again as above, fixed in PBS containing 1% paraformaldehyde, and analyzed for fluorescence on a FACSCAN cytofluorometer.

Use of increasing amounts (10 - 40 ng) of the anti-Tac antibody as competitor in the first step decreased the amount of biotinylated anti-Tac that could bind to the cells in the second step, and therefore the amount of phycoerythrin-conjugated avidin that bound in the last step, thus decreasing fluorescence (Figure 10A). Equivalent amounts (20 ng) of anti-Tac, and humanized anti-Tac used as competitor decreased the fluorescence to approximately the same degree (Figure 10B). This shows that these antibodies have approximately the same affinity (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

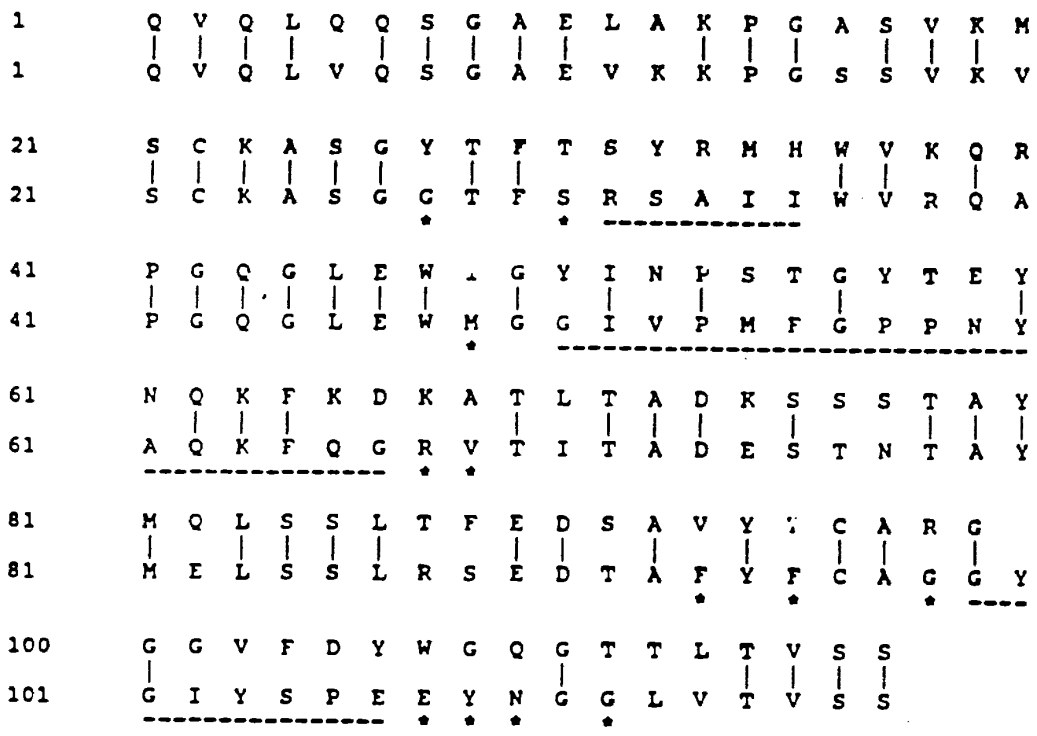
(c) the amino acid is predicted to have a side chain atom within about 3Å of the CDR's in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDR's of the humanized immunoglobulin.

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.

FIGURE 1 1/10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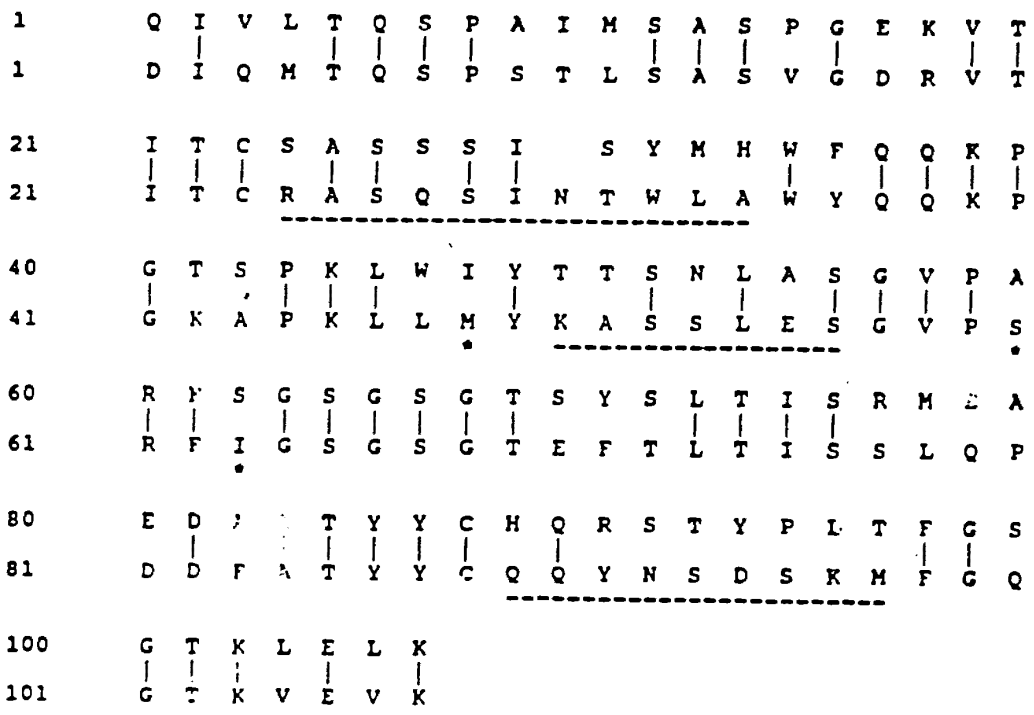
21 S C K A S G G T F S R S A I I W V R Q A
41 P G Q G L E W G Y I N P S T G Y T E Y
41 P G Q G L E W M G G I V P M F G P P N Y
61 N Q K F K D K A T L T A D K S S S T A Y
61 A Q K F Q G R V T I T A D E S T N T A Y
81 M Q L S S L T F E D S A V Y C A R G
81 M E L S S L R S E D T A F Y F C A G G Y
100 G 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

FIGURE 2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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 F T Y Y C H Q R S T Y P L T F G S
81 D D F 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

FIGURE 3

```
        10         20         30         40         50         60
TCTAGATGGGATGGAGCTGGATCTTTCTCATCCTCCTGTCAGGTACCGCGGGCGTGCACT
      M  G  W  S  W  I  F  L  F  L  L  S  G  T  A  G  V  H

        70         80         90        100        110        120
CTCAGGTCCAGCTTGTCCAGTCTGGGGCTGAAGTCAAGAAACCTGGGTCGAGCGTGAAGG
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
```

FIGURE 4

```
        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
```

FIGURE 5

A

HES12 AGCTTCTAGATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGTACCGCGGGCGTG
CACTCTCAGGTCCAGCTTGTCCAGTCTGGGGCTGAAGTCAAGAAACCTGGCTCGAGCGTG
AAGGTC

HES13 CCCAGTCGACGGATTAATATATCCAATCCATTCCAGACCCTGTCCAGGGGCCTGCCTTAC
CCAGTGCATCCTGTAGCTAGTAAAGGTGTAGCCAGAAGCCTTGCAGGAGACCTTCACGCT
CGAGCCAGG

HES14 TATATTAATCCGTCGACTGGGTATACTGAATACAATCAGAAGTTCAAGGACAAGGCAACA
ATTACTGCAGACGAATCCACCAATACAGCCTACATGGAACTGAGCAGCCTGAGATCTGAG
GACA

HES15 ATATCGTCTAGAGGTTTTAAGGACTCACCTGAGGAGACTGTGACCAGGGTTCCTTGGCCC
CAGTAGTCAAAGACCCCCCCCCCCTCTTGCACAGTAATAGACTGCGGTGTCCTCAGATCTC
AGGCTGCT

B

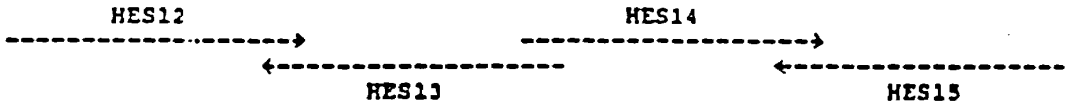


FIGURE 6 6/10

A

JFD1 CAAATCTAGATGGAGACCGATACCCTCCTGCTATGGGTCCTCCTGCTATGGGTCCCAGGA
TCAACCGGAGATATTCAGATGACCCAGTCTCCATCTACCCTCTCTGCTAGCGTCGGGGAT

JFD2 ATAAATTAGAAGCTTGGGAGCTTTGCCTGGCTTCTGCTGGTACCAGTGCATGTAACTTAT
ACTTGAGCTGGCAGAGCAGGTTATGGTGACCCTATCCCCGACGCTAGCAGAGAG

JFD3 GCTCCCAAGCTTCTAATTTATACCACATCCAACCTGGCTTCTGGAGTCCCTGCTCGCTTC
AGTGGCAGTGGATCTGGGACCGAGTTCACCCTCACAATCAGCTCTCTGCAGCCAGATGAT
TTC

JFD4 TATATCTAGAAAAGTGTACTTACGTTTGACCTCCACCTTGGTCCCCTGACCGAACGTGAG
TGGGTAAGTACTCCTTTGATGGCAGTAATAAGTGGCGAAATCATCTGGCTGCAGAGAGCT
GA

B

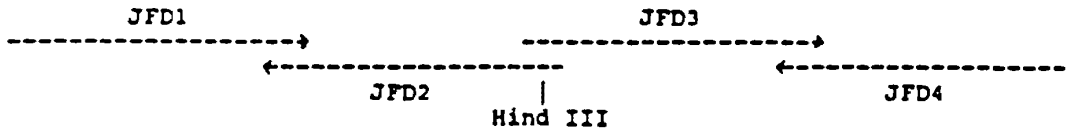


FIGURE 7

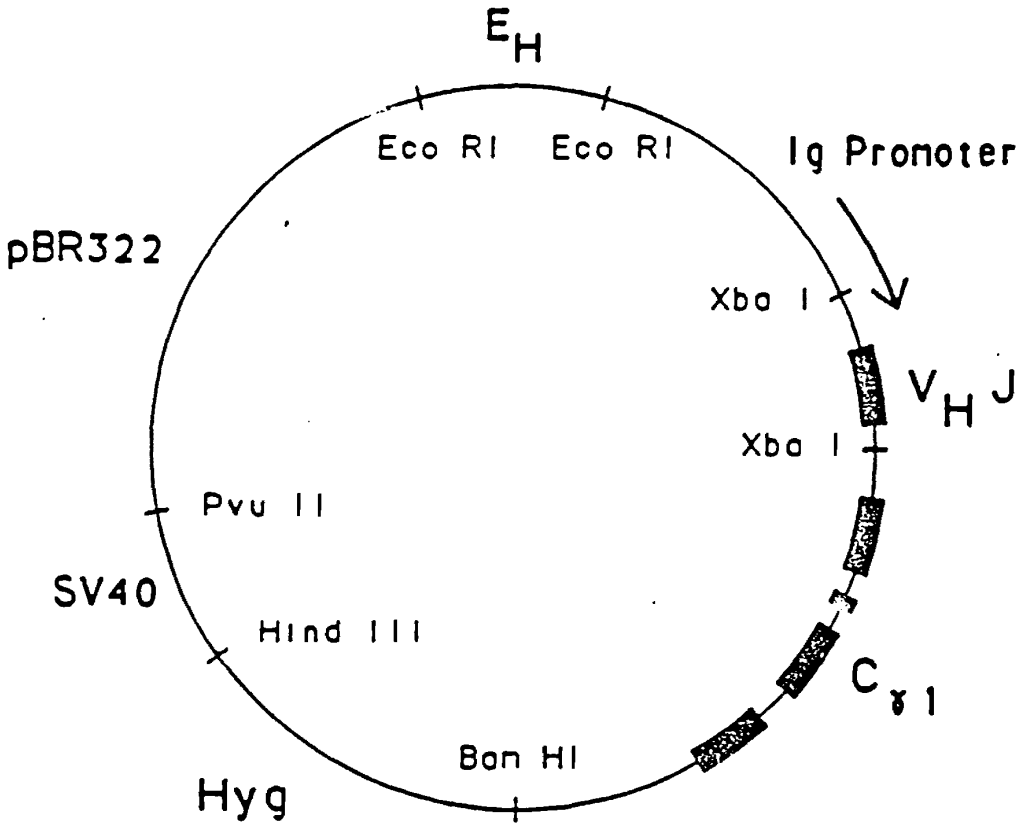

E H
Eco RI
Eco RI
Ig Promoter
pBR322
Xba I
V H J
Xba I
Pvu II
SV40
Hind III
C γ 1
Bam HI
Hyg

FIGURE 8

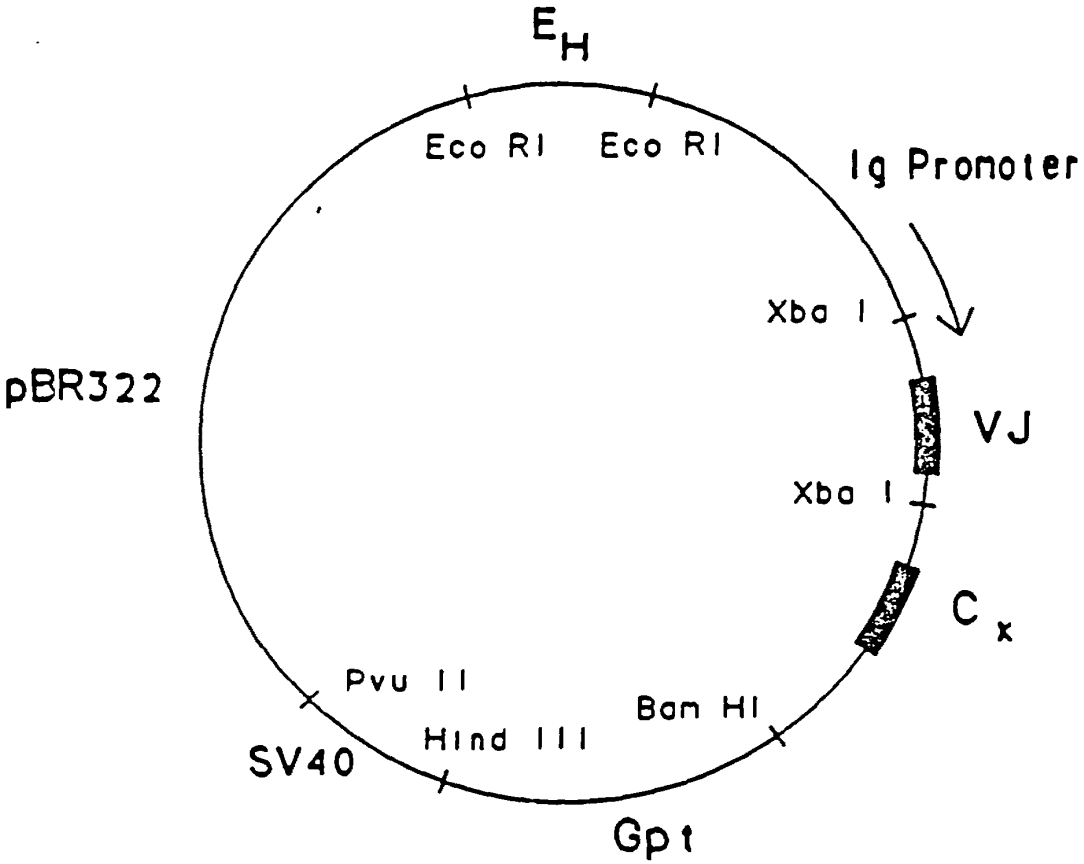

E H
Eco RI
Eco RI
Ig Promoter
Xba I
pBR322
VJ
Xba I
C x
Pvu II
Ban HI
Hind III
SV40
Gpt

FIGURE 9

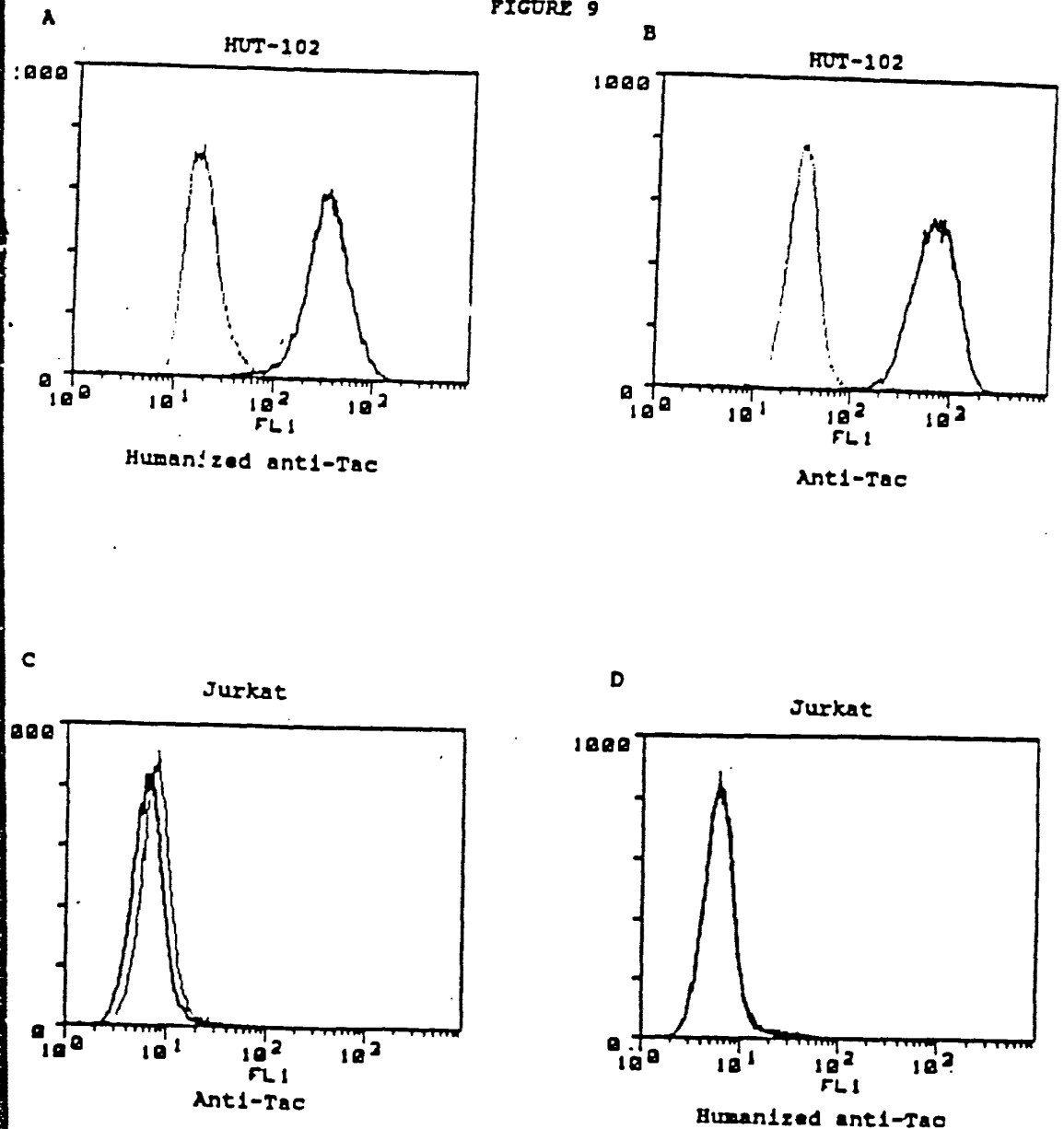
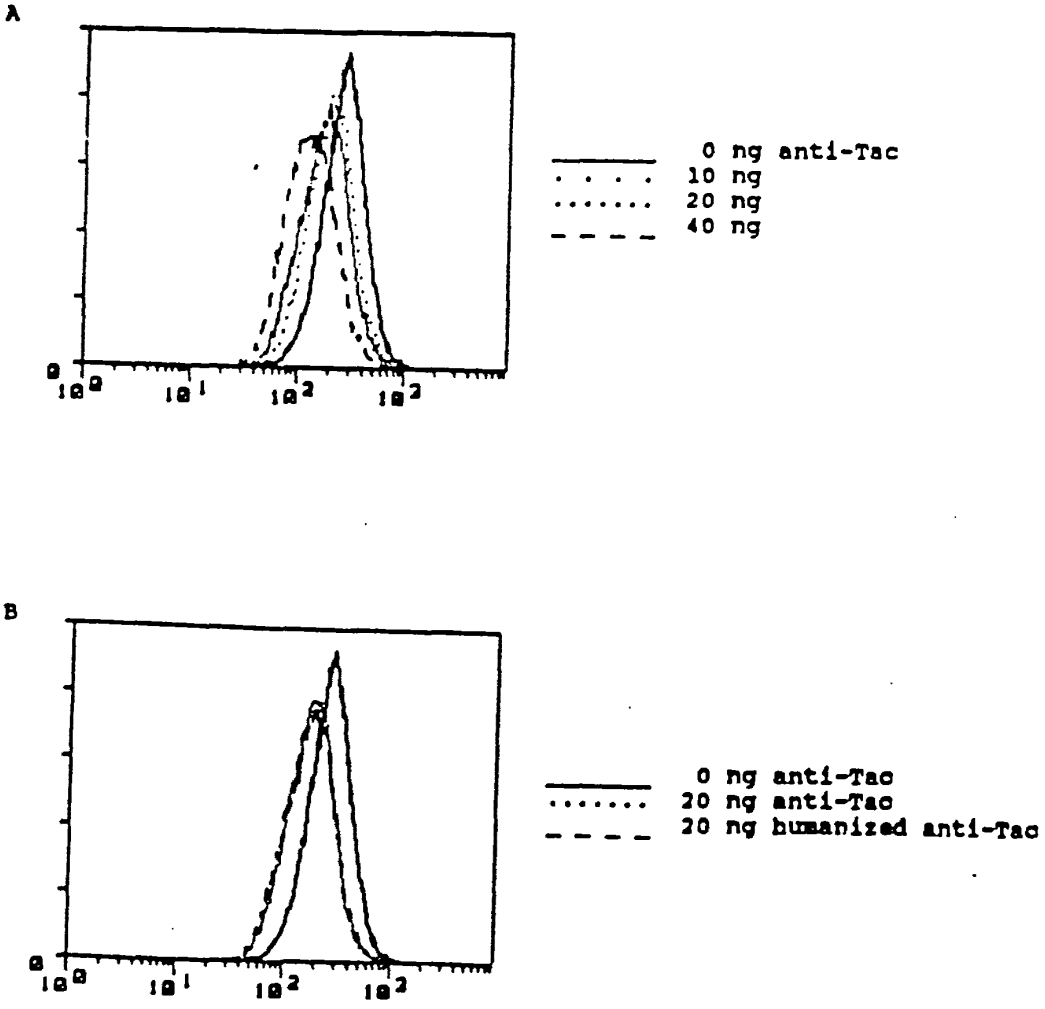

A
HUT-102
1000
0
10^0 10^1 10^2 10^2
FL1
Humanized anti-Tac
B
HUT-102
1000
0
10^0 10^1 10^2 10^2
FL1
Anti-Tac
C
Jurkat
000
0
10^0 10^1 10^2 10^2
FL1
Anti-Tac
D
Jurkat
1000
0
10^0 10^1 10^2 10^2
FL1
Humanized anti-Tac

FIGURE 10


A
0
10^0
10^1
10^2
10^3
0 ng anti-Tac
10 ng
20 ng
40 ng
B
0
10^0
10^1
10^2
10^3
0 ng anti-Tac
20 ng anti-Tac
20 ng humanized anti-Tac

INTERNATIONAL SEARCH REPORT

International Application No PCT/US89/05857

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

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

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

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
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 (VIETTA 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:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search: 01 June 1990

Date of Mailing of this International Search Report: 02 JUL 1990

International Searching Authority: ISA/US

Signature of Authorized Officer: Michelle Marks

Form PCT/ISA/210 (second sheet) (May 1986)

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 [2] 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 [3], 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 [4]

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. 4-85)

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

I. Claims 1-4, 14 and 15, drawn to a composition of [illegible] pure human-like immunoglobulin specific for p55 Tac and a method of treating T cell disorders using said immunoglobulin, classified in Class 530, subclass 387 and class 424 subclass 85.

II. Claims 5-13 and 22, drawn to a recombinant chimeric human immunoglobulin, classified in Class 435, subclass 69.1, 172.3 and class 530 subclass 387.

III. Claims 16 and 17, drawn to DNA encoding a human framework region and murine CDR of an immunoglobulin (chain?), classified in Class 536, subclass 27.

The inventions are grouped according to the unity of invention concept reflected in Rule 13.2. The inventions are distinct, each from the other because of the following reasons:

Inventions Group III and Group II are related as mutually exclusive species in intermediate-final product relationship.

Inventions Group I and Group II are related as combination and subcombination. Also, the product of Group one can be made by a non-recombinant method, which differs from the product of Group II.

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	Science, Volume 229 Issued 20 September 1985 MORRISON "Transfectomas provide novel chimeric antibodies," pp. 1202-1207. See entire document.	18-22
Y	Science, Volume 232 Issued 09 May 1986 WALDMANN "The structure, function and expression of interleukin-2 receptors on normal and malignant lymphocytes," pp. 727-732. See entire document.	1-17
X Y	Journal of Immunology, Volume 126(4) Issued 04 April 1981 UCHIYAMA "A monoclonal antibody (Anti-Tac) reactive with activated and functionally mature human T cells". See entire document.	1-4,7-8,14-15 5-6,9-13 & 16-22
X Y	Science, Volume 239 Issued 25 March 1988 VERHOEYEN ET AL "Reshaping human antibodies: grafting an antilysozyme activity," pp. 1534-1536. See entire document.	18-22 1-17
X Y	Nature, Volume 321 Issued 29 May 1986 JONES ET AL "Replacing the complementarity-determining regions in a human antibody with those from a mouse," pp. 522-525. See entire document.	18-22 1-17
X Y	Nature, Volume 332 Issued 24 March 1988 RIECHMANN ET AL "Reshaping human antibodies for therapy" pp. 323-326. See entire document.	18-22,14 1-13 & 15-17

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant *(for all designated States except US)*: CELLTECH LIMITED [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : ADAIR, John, Robert [GB/GB]; 23 George Road, Stokenchurch, High Wycombe, Buckinghamshire HP14 3RN (GB). ATHWAL, Diljeet, Singh [GB/GB]; Flat 35, Knollys House, Tavistock Square, London WC1 (GB). EMTAGE, John, Spencer [GB/GB]; 49 Temple Mill Island, Temple, Marlow, Buckinghamshire SL7 1SQ (GB).

(74) Agent: MERCER, Christopher, Paul; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

(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, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GR, 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), US.

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(54) Title: HUMANISED ANTIBODIES

(57) Abstract

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 (88) and/or (91). The CDR-grafted 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 positons (46, 48, 58) and (71). The CDR-grafted antibodies are preferably humanised antibodies, having non human, e.g. rodent, donor 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.

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HUMANISED ANTIBODIES

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" is 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 humanised 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 humanised antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanised 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 humanising MAbs by CDR-grafting was carried out 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 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 were 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 humanised 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 3×10^9 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. <u>Heavy Chain</u>

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 ($5x10^5$) 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 $5x10^5$ 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 x 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 AvaI 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-AvaI fragment. An oligonucleotide adapter was designed to replace the remainder of the 3'

region of the variable region from the AvaI site and to include the 5' residues of the human constant region up to and including a unique NarI site which had been previously engineered into the constant region.

A HindIII 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-NarI adapted fragment was purified from the ligation mixture.

The constant region was isolated as an NarI-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 HindIII 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 HindIII 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-AvaI fragment. The oligonucleotide linker was ligated to NarI 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/ClP 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 BanI 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/ClP/BanI fragment. An oligonucleotide adapter was designated to replace the remainder of the 3' region of the variable region from the BanI 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-HindIII 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/Bc11/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 -	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. <u>DISCUSSION OF CDR-GRAFTING RESULTS</u>

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	
OKT3v1	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-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 cells 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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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.

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REQUEST FOR RECTIFICATION UNDER PCT RULE 91.1(f)

Dear Sirs,

Re: International Patent Application No. PCT/GB90/02017
Celltech Limited et al.

I refer to your Invitation issued on 14th January 1991. The required Authorisations and Formal Drawings will be filed in due course.

In checking the application, it has become apparent that there are three mistakes in the Request Form.

Firstly, ...

Secondly, ...

Thirdly, for reasons which are not apparent, an old version of the Request Form (PCT/RO/101 of July 1987) was used instead of the most up-to-date version. As a result of this, some PCT states were not designated although it was the Applicant's intention that all possible states should have been designated. As evidence of this, I attach a copy of the information sheet which was given to me by hand by the Applicant's Patent Manager on the date the application

was filed. It can be seen that this clearly indicates that all territories should have been designated.

I also enclose evidence that the out-of-date Request Form was used inadvertently. At the same time as the present application was filed, I also filed two other PCT applications, Nos. PCT/GB90/02015 and PCT/GB90/02018. I enclose copies of the Request Forms for these cases which, as you can see, are the most up-to-date versions of the forms.

I therefore request that the Request Form be amended by adding thereto the designations of Canada and Spain as national applications and Greece, Spain and Denmark as designated states within the EPC designation. I note that it will not be necessary to pay any extra fees in respect of these inadvertently omitted designations.

In order to effect all these corrections, I enclose a retyped, up-to-date (at the date of filing) Request Form and request that this be substituted for the present, out-of-date Request Form.

Yours truly,

MERCER, Christopher Paul
Authorised Representative.

```
   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  MDFOVOIFSF 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
Okt3v1          QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
REI             DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK
                ? ?
                  CDR1    (LOOP)          *******
                  CDR1    (KABAT)        ***********


                          56                          85
                N  NN
RES TYPE        *IsiPpIeesesssSBEsePsPSBSSEsPspsPsseesSPePb
Okt3v1          SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
REI             APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT
                ?  ??                       ?  ?
                     *******  CDR2 (LOOP/KABAT)


                           102   108
RES TYPE        PiPIPies**iPIIsPPSPSPSS
Okt3v1          YYCQQWSSNPFTFGSGTKLEINR
REIv1           YYCQQYQSLPYTFGQGTKLQITR
                           ?    ?
                    ******        CDR3 (LOOP)
                  *********       CRD3 (KABAT)
```

Fig. 3

SUBSTITUTE SHEET

```
           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^sssssssss^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

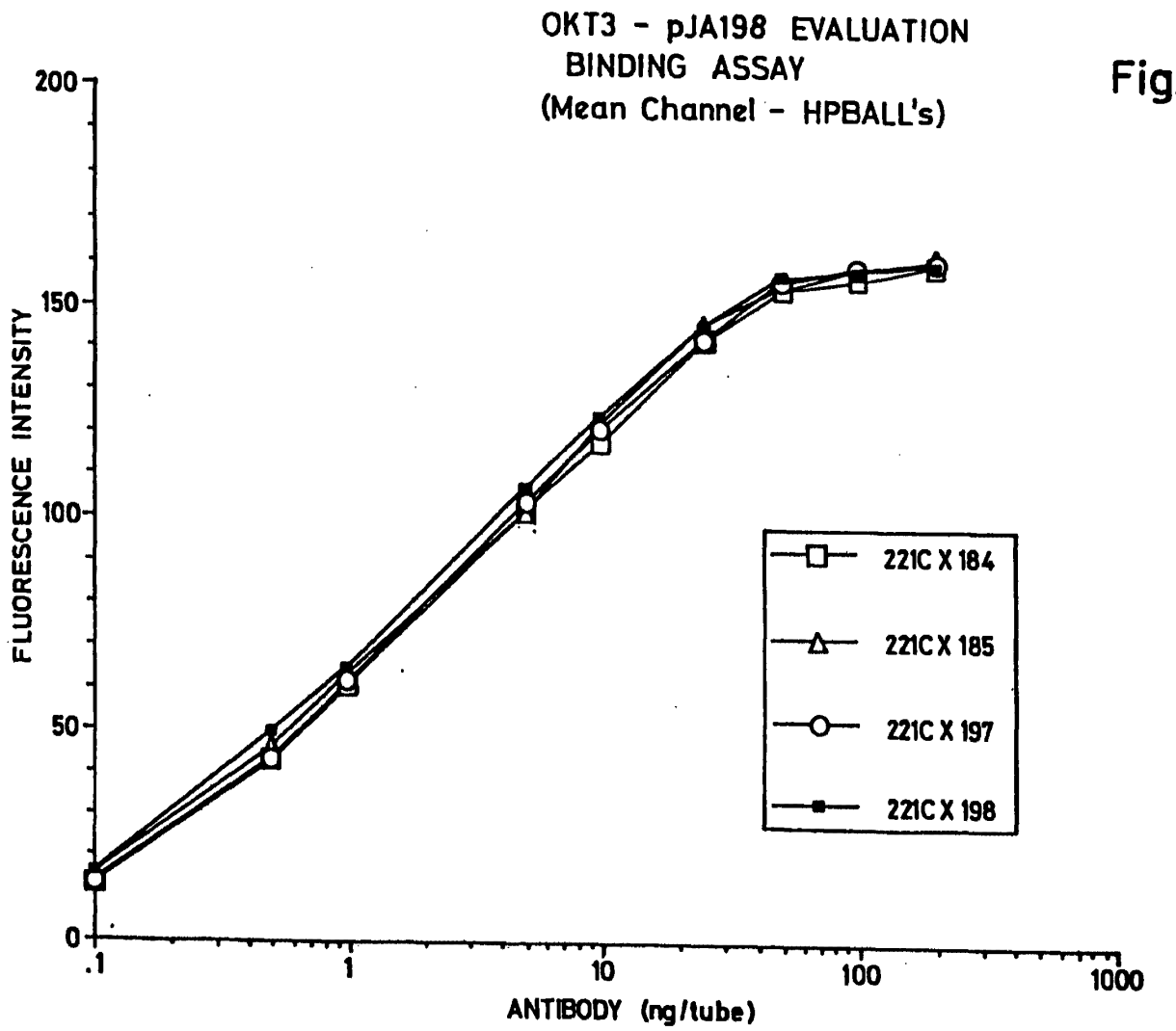
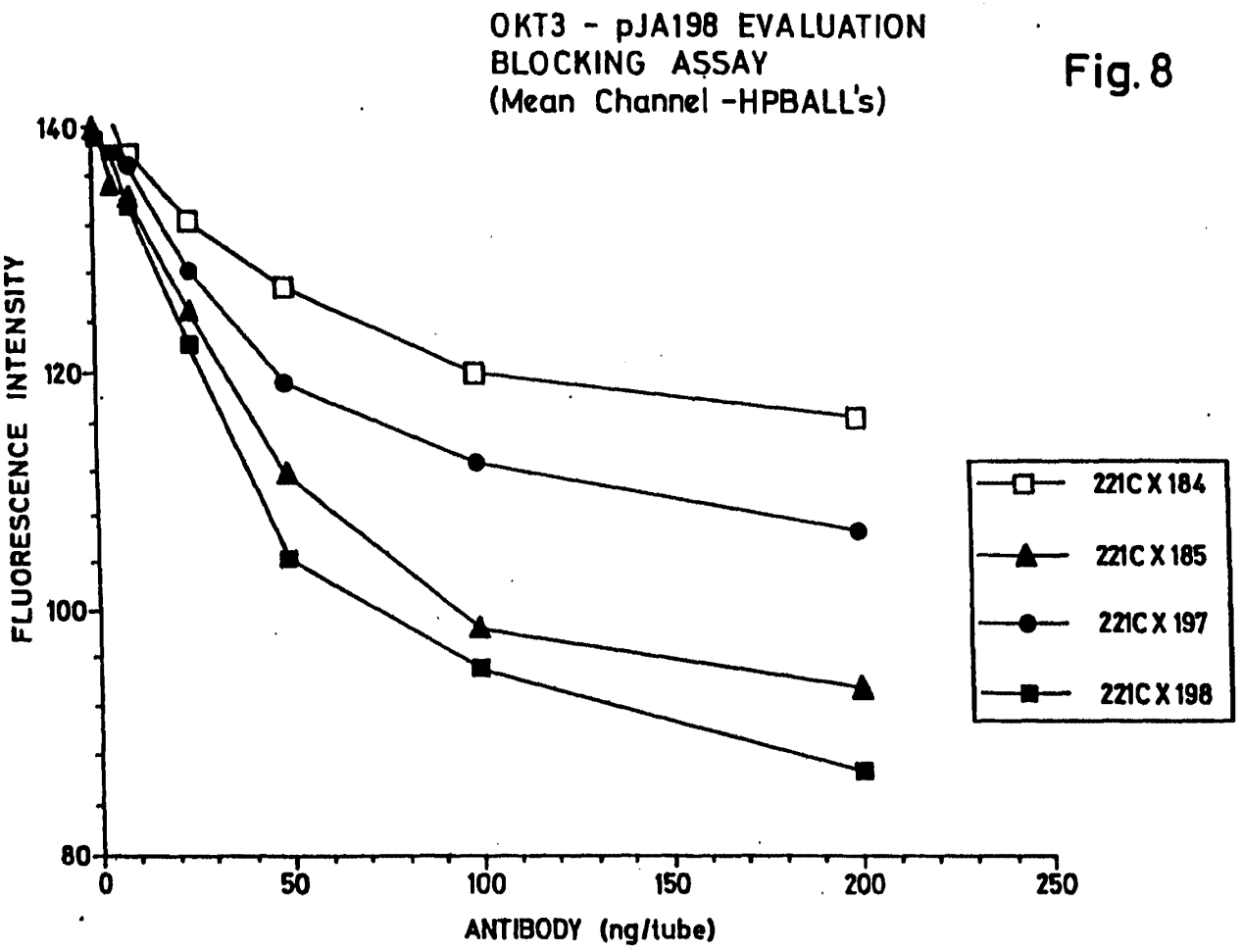


Fig. 8



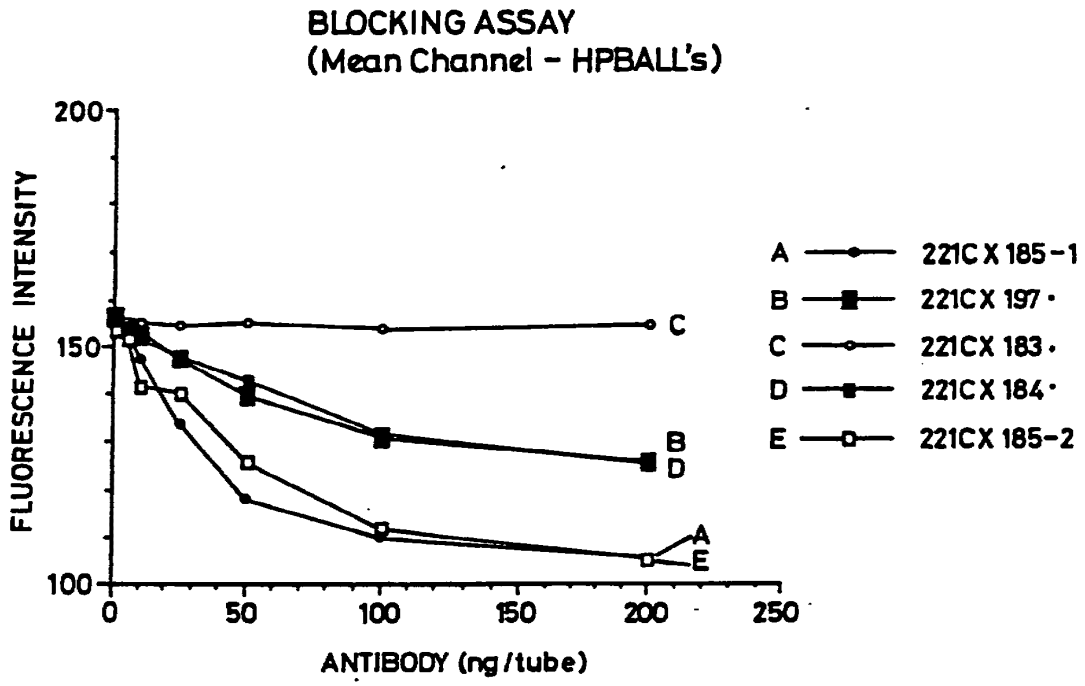
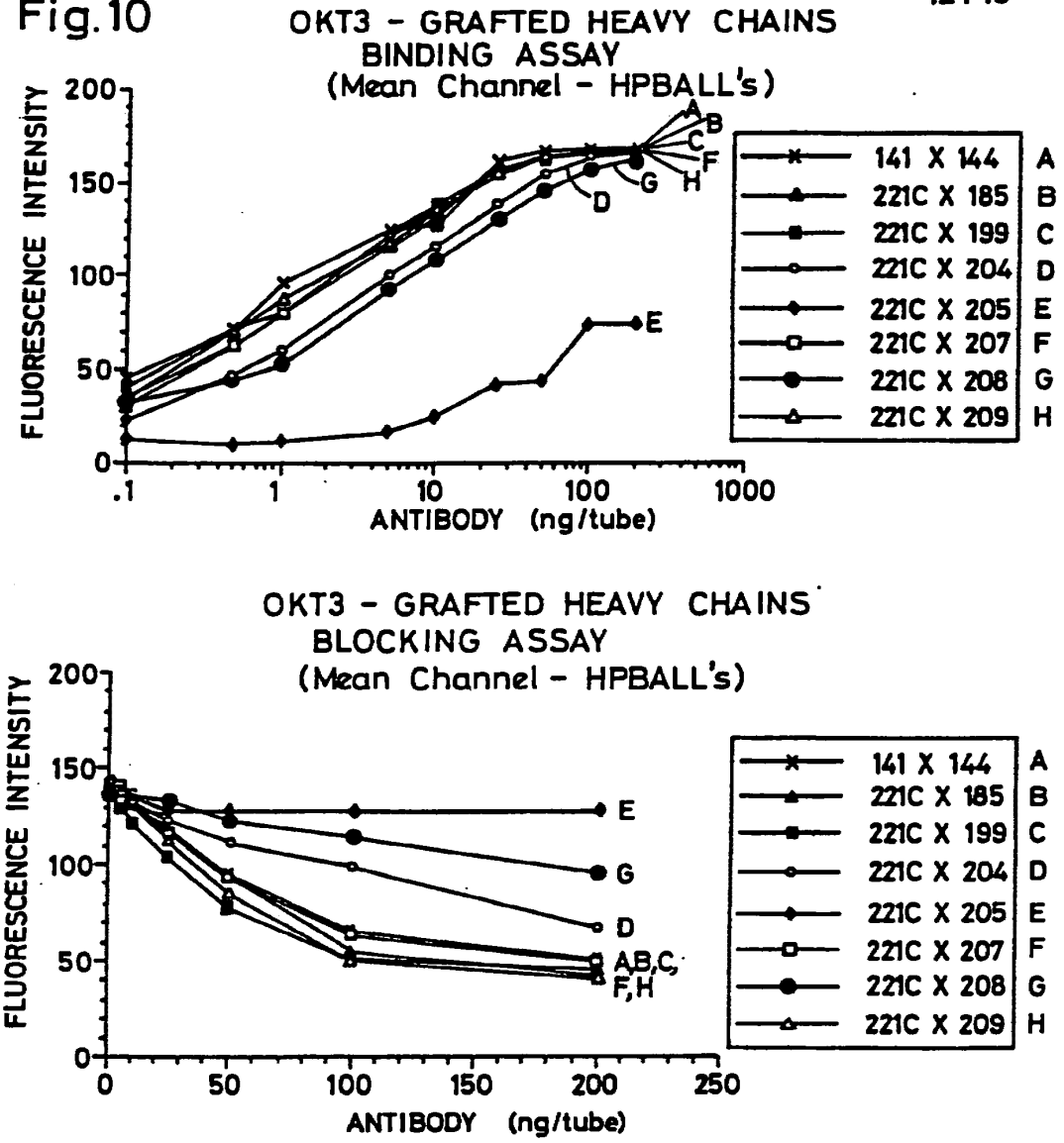

BLOCKING ASSAY
(Mean Channel - HPBALL's)
FLUORESCENCE INTENSITY
200
150
100
0
50
100
150
200
250
ANTIBODY (ng/tube)
A
B
C
D
E
A 221CX185-1
B 221CX197·
C 221CX183·
D 221CX184·
E 221CX185-2

Fig. 9

Fig.10



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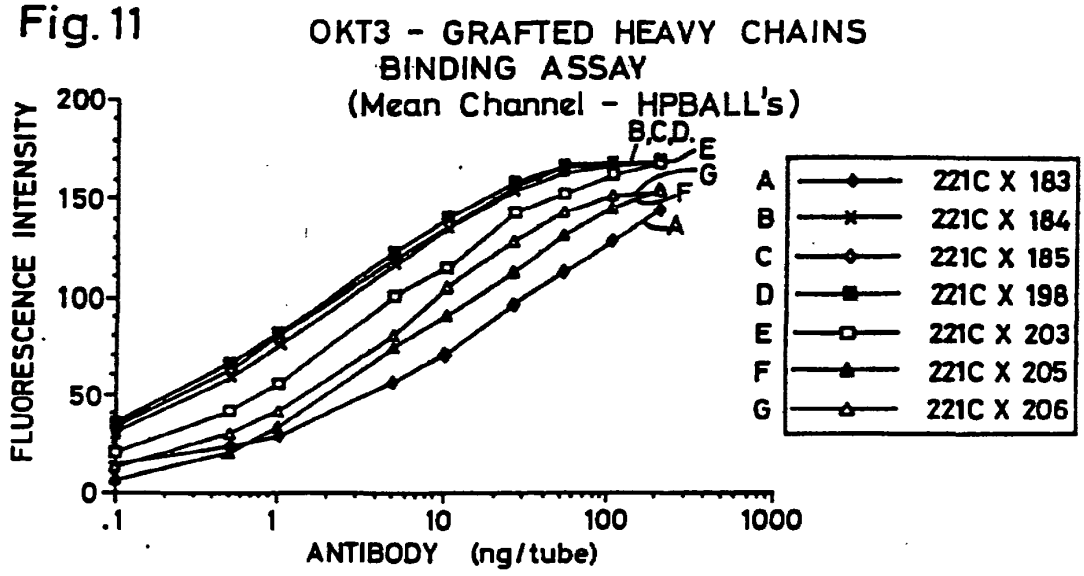
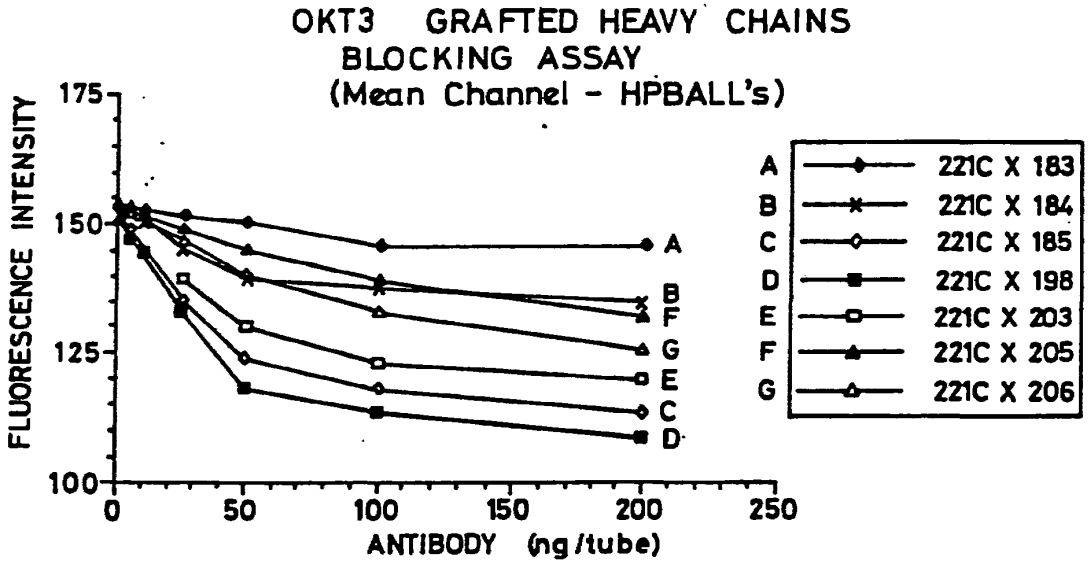
(204) 6,__,24,48,49,71,73,76,78,__,__,
(199) 6,23,24,48,49,__,__,__,__,__,__,
(207) 6,23,24,48,49,71,73,__,78,__,__,
(185) 6,23,24,48,49,71,73,76,78,88,91,
(209) 6,23,24,48,49,__,__,__,78,__,__,
141 X 144

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

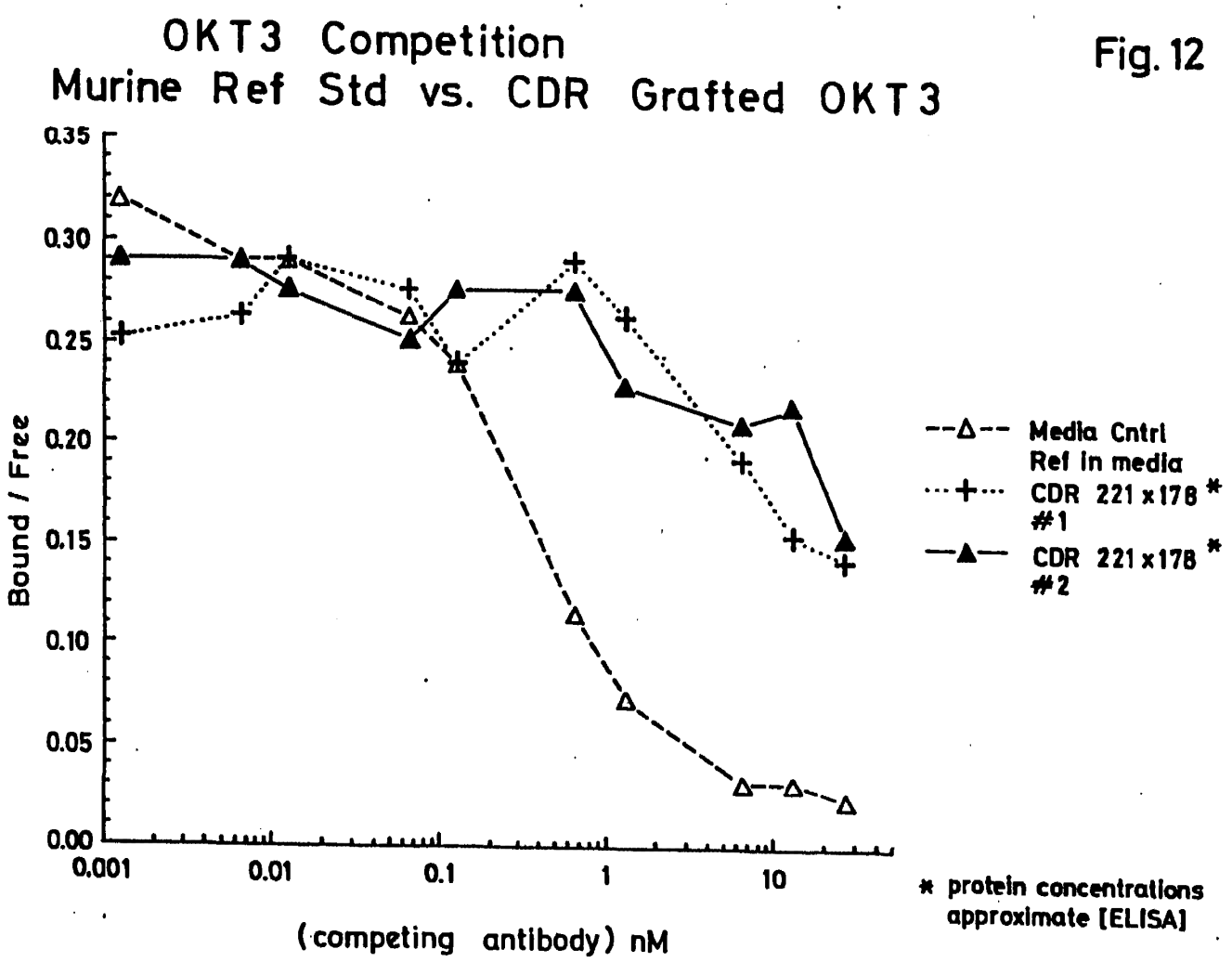
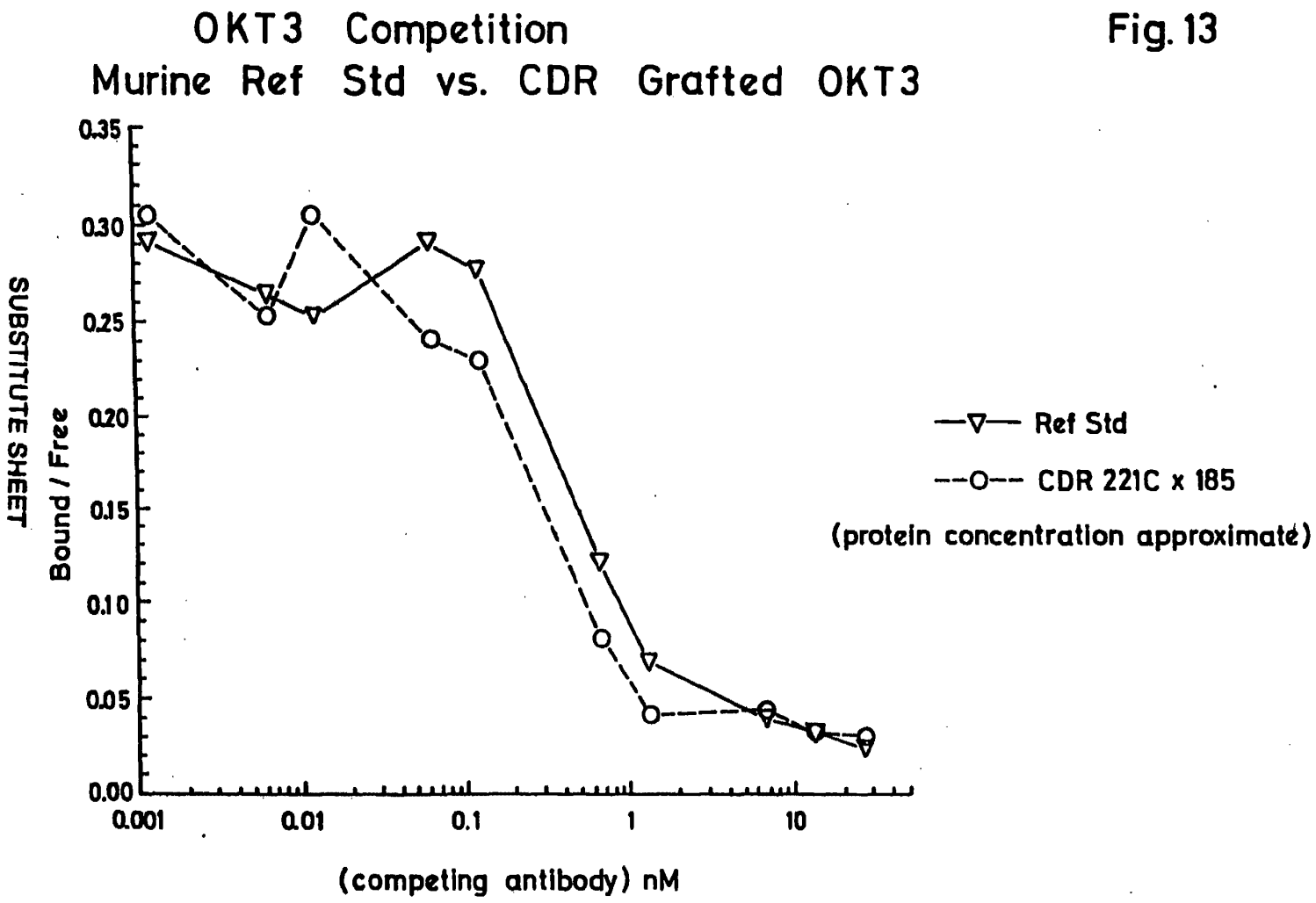

OKT3 Competition
Murine Ref Std vs. CDR Grafted OKT3
Bound / Free
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.001
0.01
0.1
1
10
(competing antibody) nM
Media Cntrl
Ref in media
CDR 221 x 178 * #1
CDR 221 x 178 * #2
* protein concentrations approximate [ELISA]

Fig. 13



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/02017

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

IPC5: C 12 P 21/08, C 12 N 15/13, A 61 K 39/395, C 07 K 15/06
C 12 N 5/10, 15/62

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 P; C 12 N; A 61 K

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

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Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	EP, A1, 0403156 (GENZYME CORPORATION ET AL.) 19 December 1990, see examples 8-12 and corresponding tables --	1,6,8, 13,14-22
Y	Proc. Natl. Acad. Sci. USA, vol. 86, December 1989, C. Queen et al.: "A humanized antibody that binds to the interleukin 2 receptor", see page 10029- page 10033 see the whole document and in particular page 10031 right col. - page 10032, left col. and page 10033 left col. --	1,6,8, 13,14-22
Y	EP, A1, 0328404 (MEDICAL RESEARCH COUNCIL ET AL.) 16 August 1989, see pages 1-3, page 9, lines 49-54 and the claims --	1,6,8, 13,14-22

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11th April 1991	17.05.91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	F.W. HECK

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

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Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature, vol. 332, March 1988, L. Riechmann et al.: "Reshaping human antibodies for therapy ", see page 323 - page 327 see in particular page 327, right col.	1,6,8, 13,144-22
A	Nature, vol. 321, May 1986, P.T. Jones et al.: "Replacing the complementarity-determining regions in a human antibody with those from a mouse ", see page 522 - page 525 see the whole document	1-22
A	Nature, vol. 328, August 1987, S. Roberts et al.: "Generation of an antibody with enhanced affinity and specificity for its antigen by protein engineering ", see page 731 - page 734 see the whole document	1,6
A	Science, vol. 239, 1988, M. Verhoeyen et al.: "Reshaping Human Antibodies: Grafting an Antilysozyme Activity ", see page 1534 - page 1536 see the whole document	1,6
A	EP, A2, 0239400 (WINTER, GREGORY PAUL) 30 September 1987, see the whole document	1,6,17-22
A	EP, A1, 0323806 (CIBA-GEIGY AG) 12 July 1989, see pages 2-6	1,6,17-22
A	Nature, vol. 341, October 1989, E.S. Ward et al.: "Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli ", see page 544 - page 546	1,6

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/02017

SA 43080

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0403156	19/12/90	NONE		
EP-A1- 0328404	16/08/89	AU-D-	3062689	06/09/89
		GB-A-	2216126	04/10/89
		WO-A-	89/07452	24/08/89
EP-A2- 0239400	30/09/87	GB-A-B-	2188638	07/10/87
		JP-A-	62296890	24/12/87
EP-A1- 0323806	12/07/89	AU-D-	2759588	06/07/89
		JP-A-	2154696	14/06/90

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/0 92/22653(1)
C07K15/00H24H-C07K15/00H34-
G06F15/00INF-[M07K201:00]-
[M07K207:00]-[M07K211:00]-

- 3- *- C07K15/00H34

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national Bureau

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

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(63) Related by Continuation
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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.
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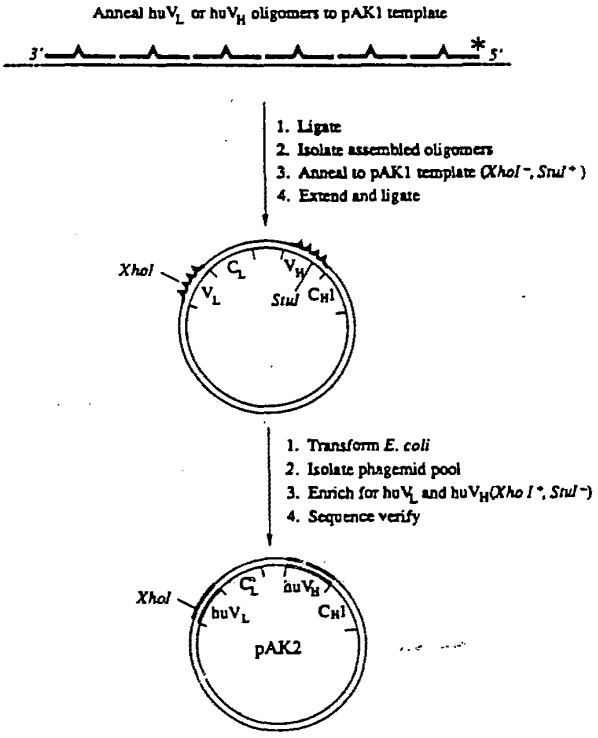
DOC

(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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METHOD FOR MAKING HUMANIZED ANTIBODIES.

Field of the Invention

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

Background of the Invention

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

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

to the formation of the antigen binding site.

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

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

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

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

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

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

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

For a given antibody a small number of FR residues are anticipated to be important for antigen binding. Firstly for example, certain antibodies have been shown to contain a few FR residues which directly contact antigen in crystal structures of antibody-antigen complexes (e.g., reviewed in Davies, D. R. *et al.*, *Ann. Rev. Biochem.* 59:439-473 (1990)). Secondly, a number of FR residues have been proposed by Chothia, Lesk and colleagues (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987); Chothia, C. *et al.*, *Nature* 342:877-883 (1989); Tramontano, A. *et al.*, *J. Mol. Biol.* 215:175-182 (1990)) as critically affecting the conformation of particular CDRs and thus their contribution to antigen binding. See also Margolies *et al.*, *Proc. Natl. Acad. Sci. USA* 72:2180-2184 (1975).

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

The three-dimensional structure of immunoglobulin chains has been studied, and crystal

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

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

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

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. *et al.*, *Cancer Res.* 50:1550-1558 (1990)), directed against the extracellular domain (ECD) of $p185^{HER2}$,

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

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

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

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

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

Summary of the Invention

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

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

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

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

d. aligning the amino acid sequences of a Framework Region (FR) of the import

antibody and the corresponding FR of the consensus antibody;

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

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

1. non-covalently binds antigen directly,

2. interacts with a CDR; or

3. participates in the V_L - V_H interface; and

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

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

Additionally, in certain embodiments the method of this invention comprises the feature wherein the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 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 (utilizing the numbering system set forth in Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)).

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

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

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

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

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

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

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

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

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

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

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLESGVP SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT

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

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTR YADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLV TVSS

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

SEQ. ID NO. 3 (light chain):

DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLESGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT, and

SEQ. ID NO. 4 (heavy chain):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENGGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTL VTVSS

Brief Description of the Drawings

FIGURE 1A shows the comparison of the V_L domain amino acid residues of muMAb4D5, huMAb4D5, and a consensus sequence (Fig. 1A, SEQ.ID NO. 5, SEQ. ID NO. 1 and SEQ. ID NO. 3, respectively). FIGURE 1B shows the comparison between the V_H domain

amino acid residues of the muMAb4d5, huMAb4D5, and a consensus sequence (Fig. 1B, SEQ. ID NO. 6, SEQ. ID NO. 2 and SEQ. ID NO. 4, respectively). Both Figs 1A and 1B use the generally accepted numbering scheme from Kabat, E. A., *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD (1987)). In both Fig. 1A and Fig. 1B, the CDR residues determined according to a standard sequence definition (as in Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) are indicated by the first underlining beneath the sequences, and the CDR residues determined according to a structural definition (as in Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987)) are indicated by the second, lower underlines. The mismatches between genes are shown by the vertical lines.

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

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

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

FIGURE 5 shows an amino acid sequence comparison of V_L (top panel) and V_H (lower panel) domains of the murine anti-CD3 monoclonal Ab UCHT1 (muxCD3, Shalaby *et al., J. Exp. Med.* 175, 217-225 (1992) with a humanized variant of this antibody (huxCD3v9). Also shown are consensus sequences (most commonly occurring residue or pair of residues) of the most abundant human subgroups, namely V_L κ 1 and V_H III upon which the humanized sequences are based (Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest*, 5th edition, National Institutes of Health, Bethesda, MD, USA (1991)). The light chain sequences--muxCD3, huxCD3v9 and huκI--correspond to SEQ.ID.NOs 16, 17, and 18, respectively. The heavy chain sequences--muxCD3, huxCD3v9 and huκI--correspond to SEQ.ID.NOs 19, 20, and 21, respectively. Residues which differ between muxCD3 and huxCD3v9 are identified by an asterisk (*), whereas those which differ between humanized and consensus sequences are identified by a sharp sign (#). A bullet (o) denotes that a residue at this position has been found to contact antigen in one or more crystallographic structures of antibody/antigen

complexes (Kabat *et al.*, 1991; Mian, I. S. *et al.*, *J. Mol. Biol.* 217, 133-151 (1991)). The location of CDR residues according to a sequence definition (Kabat *et al.*, 1991) and a structural definition (Chothia and Lesk, *supra* 1987) are shown by a line and carats (^) beneath the sequences, respectively.

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

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

Detailed Description of the Invention

Definitions

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

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

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

Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are referred to herein as "import" residues, which are typically taken from an "import" antibody domain,

particularly a variable domain. An import residue, sequence, or antibody has a desired affinity and/or specificity, or other desirable antibody biological activity as discussed herein.

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

The humanized antibody will be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG_1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG_2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art.

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

In general, humanized antibodies prepared by the method of this invention are produced by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen.

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

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

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

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

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

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

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

Throughout this description, reference is made to the numbering scheme from Kabat, E. A., *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health,

Bethesda, MD (1987) and (1991). In these compendiums, Kabat lists many amino acid sequences for antibodies for each subclass, and lists the most commonly occurring amino acid for each residue position in that subclass. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. The Kabat numbering scheme is followed in this description.

For purposes of this invention, to assign residue numbers to a candidate antibody amino acid sequence which is not included in the Kabat compendium, one follows the following steps. Generally, the candidate sequence is aligned with any immunoglobulin sequence or any consensus sequence in Kabat. Alignment may be done by hand, or by computer using commonly accepted computer programs; an example of such a program is the Align 2 program discussed in this description. Alignment may be facilitated by using some amino acid residues which are common to most Fab sequences. For example, the light and heavy chains each typically have two cysteines which have the same residue numbers; in V_L domain the two cysteines are typically at residue numbers 23 and 88, and in the V_H domain the two cysteine residues are typically numbered 22 and 92. Framework residues generally, but not always, have approximately the same number of residues, however the CDRs will vary in size. For example, in the case of a CDR from a candidate sequence which is longer than the CDR in the sequence in Kabat to which it is aligned, typically suffixes are added to the residue number to indicate the insertion of additional residues (see, e.g. residues 100abcde in Fig. 5). For candidate sequences which, for example, align with a Kabat sequence for residues 34 and 36 but have no residue between them to align with residue 35, the number 35 is simply not assigned to a residue.

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

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

to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all human immunoglobulins of any particular subclass or subunit structure. This invention provides consensus human structures and consensus structures which consider other species in addition to human.

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

Class	Heavy Chain	Subclasses	Light Chain	Molecular Formula
IgG	γ	$\gamma 1$, $\gamma 2$, $\gamma 3$, $\gamma 4$	κ or λ	$(\gamma_2\kappa_2)$, $(\gamma_2\lambda_2)$
IgA	α	$\alpha 1$, $\alpha 2$	κ or λ	$(\alpha_2\kappa_2)_n^*$, $(\alpha_2\lambda_2)_n^*$
IgM	μ	none	κ or λ	$(\mu_2\kappa_2)_5$, $(\mu_2\lambda_2)_5$
IgD	δ	none	κ or λ	$(\delta_2\kappa_2)$, $(\delta_2\lambda_2)$
IgE	ϵ	none	κ or λ	$(\epsilon_2\kappa_2)$, $(\epsilon_2\lambda_2)$

($^*_n$ may equal 1, 2, or 3)

In preferred embodiments of an IgGγ1 human consensus sequence, the consensus variable domain sequences are derived from the most abundant subclasses in the sequence compilation of Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda MD (1987), namely V_L κ subgroup I and V_H group III. In such preferred embodiments, the V_L consensus domain has the amino acid sequence: DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLESGVPSRFSG SGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT (SEQ. ID NO. 3); the V_H consensus domain has the amino acid sequence: EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENGGYTRYAD SVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLVTVSS (SEQ. ID NO. 4).

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

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

Identity or homology with respect to a specified amino acid sequence of this invention is defined herein as the percentage of amino acid residues in a candidate sequence that are

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

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

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

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

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLESGVP SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT (SEQ. ID NO. 1, which is the light chain variable domain of huMAb4D5); or

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTR YADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLV TVSS (SEQ. ID NO. 2, which is the heavy chain variable domain of huMAb4D5).

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

of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the polypeptide sequence of huMAb4D5.

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

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

An "isolated" polypeptide means polypeptide which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, for example, a polypeptide product comprising huMAb4D5 will be purified from a cell culture or other synthetic environment (1) to greater than 95% by weight of protein as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a gas- or liquid-phase sequenator (such as a commercially available Applied Biosystems sequenator Model 470, 477, or 473), or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated huMAb4D5 includes huMAb4D5 in situ within recombinant

cells since at least one component of the huMAb4D5 natural environment will not be present. Ordinarily, however, isolated huMAb4D5 will be prepared by at least one purification step.

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

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

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

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

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally,

"operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

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

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

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP 266,032 published 4 May 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, Nucl. Acids Res., 14: 5399-5407 [1986]). They are then purified on polyacrylamide gels.

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

utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

Suitable Methods for Practicing the Invention

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

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

Molecular Modeling

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

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

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

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

Table I
Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure

$V_L\kappa$ domain

Ig[a]	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus[b]
								2-11
	18-24	18-24	19-25	18-24	19-25	19-25	19-25	16-27
	32-37	34-39	39-44	32-37	32-37	32-37	33-38	33-39
								41-49
	60-66	62-68	67-72	53-66	60-65	60-65	61-66	59-77
	69-74	71-76	76-81	69-74	69-74	69-74	70-75	
	84-88	86-90	91-95	84-88	84-88	84-88	85-89	82-91
								101-105
RMS[c]		0.40	0.60	0.53	0.54	0.48	0.50	

V_H domain

Ig[a]	2FB4	2MCP	3FAB	1FBJ	2HFL	Consensus[b]
						3-8
	18-25	18-25	18-25	18-25	18-25	17-23
	34-39	34-39	34-39	34-39	34-39	33-41
	46-52	46-52	46-52	46-52	46-52	45-51
	57-61	59-63	56-60	57-61	57-61	57-61
	68-71	70-73	67-70	68-71	68-71	66-71
	78-84	80-86	77-83	78-84	78-84	75-82
	92-99	94-101	91-98	92-99	92-99	88-94
						102-108
RMS[c]		0.43	0.85	0.62	0.91	
RMS[d]	0.91	0.73	0.77	0.92		

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

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

Step 3: With the seven structures thus superimposed, for each residue in the template (2FB4) Fab one calculates the distance from the template alpha-carbon atom (Cα) to the analogous Cα atom in each of the other six superimposed structures. This results in a table of Cα-Cα distances for each residue position in the sequence. Such a table is necessary in order to determine which residue positions will be included in the consensus model. Generally, if all Cα-Cα distances for a given residue position were $\leq$ 1.0Å, that position was included in the consensus structure. If for a given position only one Fab crystal structure was > 1.0Å, the position was included but the outlying crystal structure was not included in the next step (for this position only). In general, the seven β-strands were included in the consensus structure while some of the loops connecting the β-strands, e.g. complementarity-determining regions (CDRs), were not included in view of Cα divergence.

Step 4: For each residue which was included in the consensus structure after step 3, the average of the coordinates for individual mainchain N, Cα, C, O and Cβ atoms were calculated. Due to the averaging procedure, as well as variation in bond length, bond angle and dihedral angle among the crystal structures, this "average" structure contained some bond lengths and angles which deviated from standard geometry. For purposes of this invention, "standard geometry" is understood to include geometries commonly accepted as typical, such as the compilation of bond lengths and angles from small molecule structures in Weiner, S.J. *et. al., J. Amer. Chem. Soc.*, 106: 765-784 (1984).

Step 5: In order to correct these deviations, the final step was to subject the "average" structure to 50 cycles of energy minimization (DISCOVER program, Biosym Technologies) using the AMBER (Weiner, S.J. *et. al., J. Amer. Chem. Soc.*, 106: 765-784 (1984)) parameter set with only the Cα coordinates fixed (i.e. all other atoms are allowed to move) (energy minimization is described below). This allowed any deviant bond lengths and angles to assume a standard (chemically acceptable) geometry. See Table II.

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

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

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

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

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

This consensus structure is used as the archetype. It is not particular to any species, and has only the basic shape without side chains. Starting with this consensus structure the model of *any* import, human, or humanized Fab can be constructed as follows. Using the amino acid sequence of the particular antibody VL and VH domains of interest, a computer graphics program (such as INSIGHT, Biosym Technologies) is used to add sidechains and CDRs to the consensus structure. When a sidechain is added, its conformation is chosen on the basis of known Fab crystal structures (see the Background section for publications of such crystal structures) and rotamer libraries (Ponder, J.W. & Richards, F. M., *J. Mol. Biol.* **193**: 775-791 (1987)). The model also is constructed so that the atoms of the sidechain are positioned so as to not collide with other atoms in the Fab.

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

of the canonical CDR, which is then incorporated in the evolving model.

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

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

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

Methods for Obtaining a Humanized Antibody Sequence

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

While these steps may be taken in different order, typically a structure for the candidate humanized antibody is created by transferring the at least one CDR from the non-human, import sequence into the consensus human structure, after the entire corresponding human

CDR has been removed. The humanized antibody may contain human replacements of the non-human import residues at positions within CDRs as defined by sequence variability (Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) or as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987)). For example, huMAb4D5 contains human replacements of the muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) but not as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987)): V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S.

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

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

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

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

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

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

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

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

   1. non-covalently binds antigen directly,
   2. interacts with a CDR; or
   3. participates in the V_L - V_H interface; and

g. for any non-homologous import antibody amino acid residue which is reasonably

expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

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

Additionally, in certain embodiments the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 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 (utilizing the numbering system set forth in Kabat, E. A. *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)).

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

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

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

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

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

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

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

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

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

Antibodies

Certain aspects of this invention are directed to natural antibodies and to monoclonal antibodies, as illustrated in the Examples below and by antibody hybridomas deposited with the ATCC (as described below). Thus, the references throughout this description to the use

of monoclonal antibodies are intended to include the use of natural or native antibodies as well as humanized and chimeric antibodies. As used herein, the term "antibody" includes the antibody variable domain and other separable antibody domains unless specifically excluded.

In accordance with certain aspects of this invention, antibodies to be humanized (import antibodies) are isolated from continuous hybrid cell lines formed by the fusion of antigen-primed immune lymphocytes with myeloma cells.

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

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

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for antigen titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodies are prepared by recovering immune lymphoid cells--typically spleen cells or lymphocytes from lymph node tissue--from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by

Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, *Eur. J. Immunol.* 6:511 (1976) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

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

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

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

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as Fab fragments) which bypass the generation of monoclonal antibodies are encompassed within the practice of this invention. One extracts antibody-specific messenger RNA molecules from immune system cells taken from an immunized animal,

transcribes these into complementary DNA (cDNA), and clones the cDNA into a bacterial expressions system. One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system which contains a leader sequence that causes the expressed Fab protein to migrate to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional FAb fragments for those which bind the antigen. Such FAb fragments with specificity for the antigen are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

Amino Acid Sequence Variants

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

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

A useful method for identification of certain residues or regions of the target polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science, 244: 1081-1085 [1989]).

Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the expressed target polypeptide variants are screened for the optimal combination of desired activity.

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

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

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

Other insertional variants of the target polypeptide include the fusion to the N- or C-terminus of the target polypeptide of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant

regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published 6 April 1989.

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

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

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

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

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

(3) acidic: asp, glu;

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

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

(6) aromatic: trp, tyr, phe.

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

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

of the molecule and prevent aberrant crosslinking.

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

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

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

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

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the target polypeptide, and the other strand (the original template) encodes the native, unaltered sequence of the target polypeptide. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such

as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

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

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

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

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of target polypeptide. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, *supra*, the chapter by R. Higuchi, p. 61-70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1 μg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide tri-phosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA), and 25 pmole of each oligonucleotide primer, to a final volume of 50 μl. The reaction mixture is overlayed with 35 μl mineral oil. The reaction is denatured for 5 minutes at 100°C, placed briefly on ice, and then 1 μl *Thermus aquaticus (Taq)* DNA polymerase (5 units/μl, purchased from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows: 2 min. at 55°C, then 30 sec. at 72°C, then 19 cycles of the following: 30 sec. at 94°C, 30 sec. at 55°C, and 30 sec. at 72°C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50:vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.* (Gene, 34: 315 [1985]). The starting material is the plasmid (or other vector) comprising the target polypeptide DNA to be mutated. The codon(s) in the target polypeptide DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the target polypeptide DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated target polypeptide DNA sequence.

Insertion of DNA into a Cloning Vehicle

The cDNA or genomic DNA encoding the target polypeptide is inserted into a replicable

vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(a) Signal Sequence Component

In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector.

The target polypeptides of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. Included within the scope of this invention are target polypeptides with any native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native target polypeptide signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native target polypeptide signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

(b) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal

DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of the target polypeptide DNA. However, the recovery of genomic DNA encoding the target polypeptide is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the target polypeptide DNA.

(c) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, J. Molec. Appl. Genet., 1: 327 [1982]),

mycophenolic acid (Mulligan *et al.*, Science, 209: 1422 [1980]) or hygromycin (Sugden *et al.*, Mol. Cell. Biol., 5: 410-413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the target polypeptide nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the target polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the target polypeptide are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 [1980]. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the target polypeptide. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the target polypeptide, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, Nature, 282: 39 [1979]; Kingsman *et al.*, Gene, 7: 141 [1979]; or

Tschemper *et al.*, Gene, 10: 157 [1980]). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics, 85: 12 [1977]). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

(d) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the target polypeptide nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as that encoding the target polypeptide, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the target polypeptide by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native target polypeptide promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target polypeptide DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed target polypeptide as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang *et al.*, Nature, 275: 615 [1978]; and Goeddel *et al.*, Nature, 281: 544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8: 4057 [1980] and EP 36,776) and hybrid promoters such as the tac promoter (deBoer *et al.*, Proc. Natl. Acad. Sci. USA, 80: 21-25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding the target polypeptide (Siebenlist *et al.*, Cell, 20: 269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.)

sequence operably linked to the DNA encoding the target polypeptide.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, J. Biol. Chem., 255: 2073 [1980]) or other glycolytic enzymes (Hess *et al.*, J. Adv. Enzyme Reg., 7: 149 [1968]; and Holland, Biochemistry, 17: 4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

Target polypeptide transcription from vectors in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the target polypeptide sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, Nature, 273:113 (1978); Mulligan and Berg, Science, 209: 1422-1427 (1980); Pavlakis *et al.*, Proc. Natl. Acad. Sci. USA, 78: 7398-7402 (1981). The immediate early promoter of the human

cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway *et al.*, Gene, 18: 355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is described in U.S. 4,601,978. See also Gray *et al.*, Nature, 295: 503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; , Reyes *et al.*, Nature, 297: 598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, Proc. Natl. Acad. Sci. USA, 79: 5166-5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells, and Gorman *et al.*, Proc. Natl. Acad. Sci. USA, 79: 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(e) Enhancer Element Component

Transcription of DNA encoding the target polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins *et al.*, Proc. Natl. Acad. Sci. USA, 78: 993 [1981]) and 3' (Lusky *et al.*, Mol. Cell Bio., 3: 1108 [1983]) to the transcription unit, within an intron (Banerji *et al.*, Cell, 33: 729 [1983]) as well as within the coding sequence itself (Osborne *et al.*, Mol. Cell Bio., 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the target polypeptide DNA, but is preferably located at a site 5' from the promoter.

(f) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal,

human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the target polypeptide. The 3' untranslated regions also include transcription termination sites.

Construction of suitable vectors containing one or more of the above listed components the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, Nucleic Acids Res., 9: 309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology, 65: 499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the target polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of the target polypeptide that have target polypeptide-like activity.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the target polypeptide in recombinant vertebrate cell culture are described in Gething *et al.*, Nature, 293: 620-625 [1981]; Mantei *et al.*, Nature, 281: 40-46 [1979]; Levinson *et al.*; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of the target polypeptide is pRK5 (EP pub. no. 307,247) or pSVI6B.

Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* χ1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, e.g. PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for target polypeptide-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* [Beach and Nurse, Nature, 290: 140 (1981); EP 139,383 published May 2, 1985], *Kluyveromyces* hosts (U.S. 4,943,529) such as, e.g., *K. lactis* [Louvencourt *et al.*, J. Bacteriol., 737 (1983)], *K. fragilis*, *K. bulgaricus*, *K. thermotolerans*, and *K. marxianus*, *yarrowia* [EP 402,226], *Pichia pastoris* [EP 183,070; Sreekrishna *et al.*, J. Basic Microbiol., 28: 265-278 (1988)], *Candida*, *Trichoderma reesia* [EP 244,234], *Neurospora crassa* [Case *et al.*, Proc. Natl. Acad. Sci. USA, 76: 5259-5263 (1979)], and filamentous fungi such as, e.g, *Neurospora*, *Penicillium*, *Tolypocladium* [WO 91/00357 published 10 January 1991], and *Aspergillus* hosts such as *A. nidulans* [Ballance *et al.*, Biochem. Biophys. Res. Commun., 112: 284-289 (1983); Tilburn *et al.*, Gene, 26: 205-221 (1983); Yelton *et al.*, Proc. Natl. Acad. Sci. USA, 81: 1470-1474 (1984)] and *A. niger* [Kelly and Hynes, EMBO J., 4: 475-479 (1985)].

Suitable host cells for the expression of glycosylated target polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified. See, e.g., Luckow *et al.*, Bio/Technology, 6: 47-55 (1988); Miller *et al.*, in Genetic Engineering, Setlow, J.K. *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, Nature, 315: 592-594 (1985). A variety of such viral strains are publicly

available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the target polypeptide DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding target polypeptide is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the target polypeptide DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, J. Mol. Appl. Gen., 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen Virol., 36: 59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 [1980]); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23: 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci., 383: 44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or

not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al., supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23: 315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambrook *et al, supra,* is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, J. Bact., 130: 946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA), 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

Culturing the Host Cells

Prokaryotic cells used to produce the target polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al., supra*.

The mammalian host cells used to produce the target polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz., 58: 44 (1979), Barnes and Sato, Anal. Biochem., 102: 255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements

(defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

It is further envisioned that the target polypeptides of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the target polypeptide currently in use in the field. For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired target polypeptide. The control element does not encode the target polypeptide of this invention, but the DNA is present in the host cell genome. One next screens for cells making the target polypeptide of this invention, or increased or decreased levels of expression, as desired.

Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77: 5201-5205 [1980]), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out wnere the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as

immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, Am. J. Clin. Path., 75: 734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native target polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

Purification of The Target polypeptide

The target polypeptide preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When the target polypeptide is expressed in a recombinant cell other than one of human origin, the target polypeptide is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the target polypeptide from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the target polypeptide. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The target polypeptide may then be purified from the soluble protein fraction and from the membrane fraction of the culture lysate, depending on whether the target polypeptide is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Target polypeptide variants in which residues have been deleted, inserted or substituted are recovered in the same fashion, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a target polypeptide fusion with another protein or polypeptide, e.g. a bacterial or viral antigen, facilitates purification; an

immunoaffinity column containing antibody to the antigen (or containing antigen, where the target polypeptide is an antibody) can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-target polypeptide column can be employed to absorb the target polypeptide variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native target polypeptide may require modification to account for changes in the character of the target polypeptide or its variants upon expression in recombinant cell culture.

Covalent Modifications of Target Polypeptides

Covalent modifications of target polypeptides are included within the scope of this invention. One type of covalent modification included within the scope of this invention is a target polypeptide fragment. Target polypeptide fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length target polypeptide or variant target polypeptide. Other types of covalent modifications of the target polypeptide or fragments thereof are introduced into the molecule by reacting specific amino acid residues of the target polypeptide or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include

imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking target polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-target polypeptide antibodies, and vice versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the target polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in the native target polypeptide, and/or adding one or more glycosylation sites that are not present in the native target polypeptide.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tri-peptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose, to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the target polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native target polypeptide sequence (for O-linked glycosylation sites). For ease, the target polypeptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the target polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of Target Polypeptide".

Another means of increasing the number of carbohydrate moieties on the target polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- and O- linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free

carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston (CRC Crit. Rev. Biochem., pp. 259-306 [1981]).

Removal of carbohydrate moieties present on the native target polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin *et al.* (Arch. Biochem. Biophys., 259:52 [1987]) and by Edge *et al.* (Anal. Biochem., 118:131 [1981]). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.* (Meth. Enzymol., 138:350 [1987]).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin *et al.* (J. Biol. Chem., 257:3105 [1982]). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of the target polypeptide comprises linking the target polypeptide to various nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The target polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate] microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., (1980).

Target polypeptide preparations are also useful in generating antibodies, for screening for binding partners, as standards in assays for the target polypeptide (e.g. by labeling the target polypeptide for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant target polypeptide, it will be appreciated that some screening of the recovered variant will be needed

to select the optimal variant. For example, a change in the immunological character of the target polypeptide molecule, such as affinity for a given antigen or antibody, is measured by a competitive-type immunoassay. The variant is assayed for changes in the suppression or enhancement of its activity by comparison to the activity observed for the target polypeptide in the same assay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability in recombinant cell culture or in plasma, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

Diagnostic and Related Uses of the Antibodies

The antibodies of this invention are useful in diagnostic assays for antigen expression in specific cells or tissues. The antibodies are detectably labeled and/or are immobilized on an insoluble matrix.

The antibodies of this invention find further use for the affinity purification of the antigen from recombinant cell culture or natural sources. Suitable diagnostic assays for the antigen and its antibodies depend on the particular antigen or antibody. Generally, such assays include competitive and sandwich assays, and steric inhibition assays. Competitive and sandwich methods employ a phase-separation step as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of the antigen and for substances that bind the antigen, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors, or antigens.

Analytical methods for the antigen or its antibodies all use one or more of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner and steric conjugates. The labeled reagents also are known as "tracers."

The label used (and this is also useful to label antigen nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized

to be detected. Examples of such labels include the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalently to proteins or polypeptides. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. Nos. 3,940,475 (fluorimetry) and 3,645,090 (enzymes); Hunter *et al.*, Nature, 144: 945 (1962); David *et al.*, Biochemistry, 13: 1014-1021 (1974); Pain *et al.*, J. Immunol. Methods, 40: 219-230 (1981); and Nygren, J. Histochem. and Cytochem., 30: 407-412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase.

The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan *et al.*, "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in Methods in Enzymology, ed. J.J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, New York, 1981), pp. 147-166. Such bonding methods are suitable for use with the antibodies and polypeptides of this invention.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich *et al.*., U.S. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analogue afterward, e.g., by immunoprecipitation.

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding

partner generally is insolubilized before or after the competition and then the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared and used such that when anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, the antigen or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with antibody so that binding of the antibody inhibits or potentiates the enzyme activity of the label. This method *per se* is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in fluorescence when the hapten is a fluorophore.

Sandwich assays particularly are useful for the determination of antigen or antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled binding partner. A sequential sandwich assay using an anti-antigen monoclonal antibody as one antibody and a polyclonal anti-antigen antibody as the other is useful in testing samples for particular antigen activity.

The foregoing are merely exemplary diagnostic assays for the import and humanized antibodies of this invention. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described

above.

Immunotoxins

This invention is also directed to immunochemical derivatives of the antibodies of this invention such as immunotoxins (conjugates of the antibody and a cytotoxic moiety). Antibodies which carry the appropriate effector functions, such as with their constant domains, are also used to induce lysis through the natural complement process, and to interact with antibody dependent cytotoxic cells normally present.

For example, purified, sterile filtered antibodies are optionally conjugated to a cytotoxin such as ricin for use in AIDS therapy. US Patent Application Serial No. 07/350,895 illustrates methods for making and using immunotoxins for the treatment of HIV infection. The methods of this invention, for example, are suitable for obtaining humanized antibodies for use as immunotoxins for use in AIDS therapy.

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal, plant or animal origin, or an enzymatically active fragment of such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. In another embodiment, the antibodies are conjugated to small molecule anticancer drugs such as cis-platin or 5FU. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT , bifunctional derivatives of imidoesters such as dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis- (p-diazoniumbenzoyl)- -ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate and bis-active fluorine compounds such as 1,5-difluoro- 2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

Immunotoxins can be made in a variety of ways, as discussed herein. Commonly known crosslinking reagents can be used to yield stable conjugates.

Advantageously, monoclonal antibodies specifically binding the domain of the antigen which is exposed on the infected cell surface, are conjugated to ricin A chain. Most

advantageously the ricin A chain is deglycosylated and produced through recombinant means. An advantageous method of making the ricin immunotoxin is described in Vitetta *et al.*, *Science* 238:1098 (1987).

When used to kill infected human cells *in vitro* for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for *in vitro* use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques.

Cytotoxic radiopharmaceuticals for treating infected cells may be made by conjugating radioactive isotopes (e.g. I, Y, Pr) to the antibodies. Advantageously alpha particle-emitting isotopes are used. The term 'cytotoxic moiety" as used herein is intended to include such isotopes.

In a preferred embodiment, ricin A chain is deglycosylated or produced without oligosaccharides, to decrease its clearance by irrelevant clearance mechanisms (e.g., the liver). In another embodiment, whole ricin (A chain plus B chain) is conjugated to antibody if the galactose binding property of B-chain can be blocked ("blocked ricin").

In a further embodiment toxin-conjugates are made with Fab or $F(ab')_2$ fragments. Because of their relatively small size these fragments can better penetrate tissue to reach infected cells.

In another embodiment, fusogenic liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding the particular antigen.

Antibody Dependent Cellular Cytotoxicity

Certain aspects of this invention involve antibodies which are (a) directed against a particular antigen and (b) belong to a subclass or isotype that is capable of mediating the lysis of cells to which the antibody molecule binds. More specifically, these antibodies should belong to a subclass or isotype that, upon complexing with cell surface proteins, activates serum complement and/or mediates antibody dependent cellular cytotoxicity (ADCC) by activating effector cells such as natural killer cells or macrophages.

Biological activity of antibodies is known to be determined, to a large extent, by the constant domains or Fc region of the antibody molecule (Uananue and Benacerraf, *Textbook of Immunology*, 2nd Edition, Williams & Wilkins, p. 218 (1984)). This includes their ability to activate complement and to mediate antibody-dependent cellular cytotoxicity (ADCC) as effected by leukocytes. Antibodies of different classes and subclasses differ in this respect,

as do antibodies from the same subclass but different species; according to the present invention, antibodies of those classes having the desired biological activity are prepared. Preparation of these antibodies involves the selection of antibody constant domains are their incorporation in the humanized antibody by known technique. For example, mouse immunoglobulins of the IgG3 and IgG2a class are capable of activating serum complement upon binding to the target cells which express the cognate antigen, and therefore humanized antibodies which incorporate IgG3 and IgG2a effector functions are desirable for certain therapeutic applications.

In general, mouse antibodies of the IgG2a and IgG3 subclass and occasionally IgG1 can mediate ADCC, and antibodies of the IgG3, IgG2a, and IgM subclasses bind and activate serum complement. Complement activation generally requires the binding of at least two IgG molecules in close proximity on the target cell. However, the binding of only one IgM molecule activates serum complement.

The ability of any particular antibody to mediate lysis of the target cell by complement activation and/or ADCC can be assayed. The cells of interest are grown and labeled *in vitro*; the antibody is added to the cell culture in combination with either serum complement or immune cells which may be activated by the antigen antibody complexes. Cytolysis of the target cells is detected by the release of label from the lysed cells. In fact, antibodies can be screened using the patient's own serum as a source of complement and/or immune cells. The antibody that is capable of activating complement or mediating ADCC in the *in vitro* test can then be used therapeutically in that particular patient.

This invention specifically encompasses consensus Fc antibody domains prepared and used according to the teachings of this invention.

Therapeutic and Other Uses of the Antibodies

When used *in vivo* for therapy, the antibodies of the subject invention are administered to the patient in therapeutically effective amounts (i.e. amounts that have desired therapeutic effect). They will normally be administered parenterally. The dose and dosage regimen will depend upon the degree of the infection, the characteristics of the particular antibody or immunotoxin used, e.g., its therapeutic index, the patient, and the patient's history. Advantageously the antibody or immunotoxin is administered continuously over a period of 1-2 weeks, intravenously to treat cells in the vasculature and subcutaneously and intraperitoneally to treat regional lymph nodes. Optionally, the administration is made during the course of adjunct therapy such as combined cycles of radiation, chemotherapeutic treatment, or

administration of tumor necrosis factor, interferon or other cytoprotective or immunomodulatory agent.

For parenteral administration the antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic, and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate can also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The antibodies will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

Use of IgM antibodies may be preferred for certain applications, however IgG molecules by being smaller may be more able than IgM molecules to localize to certain types of infected cells.

There is evidence that complement activation *in vivo* leads to a variety of biological effects, including the induction of an inflammatory response and the activation of macrophages (Uananue and Benecerraf, *Textbook of Immunology*, 2nd Edition, Williams & Wilkins, p. 218 (1984)). The increased vasodilation accompanying inflammation may increase the ability of various agents to localize in infected cells. Therefore, antigen-antibody combinations of the type specified by this invention can be used therapeutically in many ways. Additionally, purified antigens (Hakomori, *Ann. Rev. Immunol.* 2:103 (1984)) or anti-idiotypic antibodies (Nepom *et al.*, *Proc. Natl. Acad. Sci.* 81:2864 (1985); Koprowski *et al.*, *Proc. Natl. Acad. Sci.* 81:216 (1984)) relating to such antigens could be used to induce an active immune response in human patients. Such a response includes the formation of antibodies capable of activating human complement and mediating ADCC and by such mechanisms cause infected cell destruction.

Optionally, the antibodies of this invention are useful in passively immunizing patients, as exemplified by the administration of humanized anti-HIV antibodies.

The antibody compositions used in therapy are formulated and dosages established in a fashion consistent with good medical practice taking into account the disorder to be treated, the condition of the individual patient, the site of delivery of the composition, the method of administration and other factors known to practitioners. The antibody compositions are prepared for administration according to the description of preparation of polypeptides for administration, *infra*.

Deposit of Materials

As described above, cultures of the muMAb4D5 have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC).

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of the deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.12 with particular reference to 886 OG 638).

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the constructs deposited, since the deposited embodiments are intended to illustrate only certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those

skilled in the art from the foregoing description and fall within the scope of the appended claims.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention.

EXAMPLES

EXAMPLE 1. HUMANIZATION OF muMAb4D5

Here we report the chimerization of muMAb4D5 (chMAb4D5) and the rapid and simultaneous humanization of heavy (V_H) and light (V_L) chain variable region genes using a novel "gene conversion mutagenesis" strategy. Eight humanized variants (huMAb4D5) were constructed to probe the importance of several FR residues identified by our molecular modeling or previously proposed to be critical to the conformation of particular CDRs (see Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987); Chothia, C. *et al., Nature* 342:877-883 (1989); Tramontano, A. *et al., J. Mol. Biol.* 215:175-182 (1990)). Efficient transient expression of humanized variants in non-myeloma cells allowed us to rapidly investigate the relationship between binding affinity for $p185^{HER2}$ ECD and anti-proliferative activity against $p185^{HER2}$ overexpressing carcinoma cells.

MATERIALS and METHODS

Cloning of Variable Region Genes. The muMAb4D5 V_H and V_L genes were isolated by polymerase chain reaction (PCR) amplification of mRNA from the corresponding hybridoma (Fendly, B. M. *et al., Cancer Res.* 50:1550-1558 (1990)) as described by Orlandi et al. (Orlandi, R. *et al., Proc. Natl. Acad. Sci. USA* 86:3833-3837 (1989)). Amino terminal sequencing of muMAb4D5 V_L and V_H was used to design the sense strand PCR primers, whereas the anti-sense PCR primers were based upon consensus sequences of murine framework residues (Orlandi, R. *et al., Proc. Natl. Acad. Sci. USA* 86:3833-3837 (1989); Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) incorporating restriction sites for directional cloning shown by underlining and listed after the sequences: V_L sense, 5'-TCCGATATCCAGCTGACCCAGTCTCCA-3' (SEQ. ID NO. 7), *Eco*RV; V_L anti-sense, 5'-

GTTTGATCTCCAGCTTGGTACCHSCDCCGAA-3' (SEQ. ID NO. 8), *Asp*718; V_H sense, 5'-AGGTSMARCTGCAGSAGTCWGG-3' (SEQ. ID NO. 9), *Pst*I and V_H anti-sense, 5'-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG-3' (SEQ. ID NO. 10), *Bst*EII; where H = A or C or T, S = C or G, D = A or G or T, M = A or C, R = A or G and W = A or T. The PCR products were cloned into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3-11 (1987)) and five clones for each variable domain sequenced by the dideoxy method (Sanger, F. *et al.*, *Proc. Natl. Acad. Sci. USA* 74:5463-5467 (1977)).

Molecular Modelling. Models for muMAb4D5 V_H and V_L domains were constructed separately from consensus coordinates based upon seven Fab structures from the Brookhaven protein data bank (entries 1FB4, 2RHE, 2MCP, 3FAB, 1FBJ, 2HFL and 1REI). The Fab fragment KOL (Marquart, M. *et al.*, *J. Mol. Biol.* 141:369-391 (1980)) was first chosen as a template for V_L and V_H domains and additional structures were then superimposed upon this structure using their main chain atom coordinates (INSIGHT program, Biosym Technologies). The distance from the template Cα to the analogous Cα in each of the superimposed structures was calculated for each residue position. If all (or nearly all) Cα-Cα distances for a given residue were $\leq$ 1Å, then that position was included in the consensus structure. In most cases the β-sheet framework residues satisfied these criteria whereas the CDR loops did not. For each of these selected residues the average coordinates for individual N, Cα, C, O and Cβ atoms were calculated and then corrected for resultant deviations from non-standard bond geometry by 50 cycles of energy minimization using the DISCOVER program (Biosym Technologies) with the AMBER forcefield (Weiner, S. J. *et al.*, *J. Amer. Chem. Soc.* 106:765-784 (1984)) and Cα coordinates fixed. The side chains of highly conserved residues, such as the disulfide-bridged cysteine residues, were then incorporated into the resultant consensus structure. Next the sequences of muMAb4D5 V_L and V_H were incorporated starting with the CDR residues and using the tabulations of CDR conformations from Chothia et al. (Chothia, C. *et al.*, *Nature* 342:877-883 (1989)) as a guide. Side-chain conformations were chosen on the basis of Fab crystal structures, rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193:775-791 (1987)) and packing considerations. Since V_H-CDR3 could not be assigned a definite backbone conformation from these criteria, two models were created from a search of similar sized loops using the INSIGHT program. A third model was derived using packing and solvent exposure considerations. Each model was then subjected to 5000 cycles of energy minimization.

In humanizing muMAb4D5, consensus human sequences were first derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. *et al.*,

Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, MD, 1987)), namely V_L κ subgroup I and V_H group III, and a molecular model generated for these sequences using the methods described above. A structure for huMAb4D5 was created by transferring the CDRs from the muMAb4D5 model into the consensus human structure. All huMAb4D5 variants contain human replacements of muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) but not as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* **196**:901-917 (1987)): V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S. Differences between muMAb4D5 and the human consensus framework residues (Fig. 1) were individually modeled to investigate their possible influence on CDR conformation and/or binding to the $p185^{HER2}$ ECD.

Construction of Chimeric Genes. Genes encoding chMAb4D5 light and heavy chains were separately assembled in previously described phagemid vectors containing the human cytomegalovirus enhancer and promoter, a 5' intron and SV40 polyadenylation signal (Gorman, C. M. *et al.*, *DNA & Prot. Engin. Tech.* 2:3-10 (1990)). Briefly, gene segments encoding muMAb4D5 V_L (Fig. 1A) and REI human κ_1 light chain C_L (Palm, W. & Hilschmann, N., *Z. Physiol. Chem.* **356**:167-191 (1975)) were precisely joined as were genes for muMAb4D5 V_H (Fig. 1B) and human γ1 constant region (Capon, D. J. *et al.*, *Nature* **337**:525-531 (1989)) by simple subcloning (Boyle, A., in *Current Protocols in Molecular Biology*, Chapter 3 (F. A. Ausubel *et al.*, eds., Greene Publishing & Wiley-Interscience, New York, 1990)) and site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The γ1 isotype was chosen as it has been found to be the preferred human isotype for supporting ADCC and complement dependent cytotoxicity using matched sets of chimeric (Brüggemann, M. *et al.*, *J. Exp. Med.* **166**:1351-1361 (1987)) or humanized antibodies (Riechmann, L. *et al.*, *Nature* **332**:323-327 (1988)). The PCR-generated V_L and V_H fragments (Fig. 1) were subsequently mutagenized so that they faithfully represent the sequence of muMAb4D5 determined at the protein level: V_H Q1E, V_L V104L and T109A (variants are denoted by the amino acid residue and number followed by the replacement amino acid). The human γ1 constant regions are identical to those reported by Ellison et al. (Ellison, J. W. *et al.*, *Nucleic Acids Res.* 13:4071-4079 (1982)) except for the mutations E359D and M361L (Eu numbering, as in Kabat, E. A. *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) which we installed to convert the antibody from the naturally rare A allotype to the much more common non-A allotype (Tramontano, A. *et al.*, *J. Mol. Biol.* 215:175-182 (1990)). This was an attempt to

reduce the risk of anti-allotype antibodies interfering with therapy.

Construction of Humanized Genes. Genes encoding chMAb4D5 light chain and heavy chain Fd fragment (V_H and C_H1 domains) were subcloned together into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* **153**:3-11 (1987)) to create pAK1 and simultaneously humanized in a single step (Fig. 2). Briefly, sets of 6 contiguous oligonucleotides were designed to humanize V_H and V_L (Fig. 1). These oligonucleotides are 28 to 83 nucleotides in length, contain zero to 19 mismatches to the murine antibody template and are constrained to have 8 or 9 perfectly matched residues at each end to promote efficient annealing and ligation of adjacent oligonucleotides. The sets of V_H and V_L humanization oligonucleotides (5 pmol each) were phosphorylated with either ATP or γ-^{32}P-ATP (Carter, P. *Methods Enzymol.* **154**:382-403 (1987)) and separately annealed with 3.7 pmol of pAK1 template in 40 μl 10 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ by cooling from 100 °C to room temperature over ~30 min. The annealed oligonucleotides were joined by incubation with T4 DNA ligase (12 units; New England Biolabs) in the presence of 2 μl 5 mM ATP and 2 μl 0.1 M DTT for 10 min at 14 °C. After electrophoresis on a 6% acrylamide sequencing gel the assembled oligonucleotides were located by autoradiography and recovered by electroelution. The assembled oligonucleotides (~0.3 pmol each) were simultaneously annealed to 0.15 pmol single-stranded deoxyuridine-containing pAK1 prepared according to Kunkel et al. (Kunkel, T. A. *et al., Methods Enzymol.* **154**:367-382 (1987)) in 10 μl 40 mM Tris-HCl (pH 7.5) and 16 mM $MgCl_2$ as above. Heteroduplex DNA was constructed by extending the primers with T7 DNA polymerase and transformed into *E. coli* BMH 71-18 *mut*L as previously described (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The resultant phagemid DNA pool was enriched first for huV_L by restriction purification using *Xho*I and then for huV_H by restriction selection using *Stu*I as described in Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991); and in Wells, J. A. *et al., Phil. Trans. R. Soc. Lond. A* **317**:415-423 (1986). Resultant clones containing both huV_L and huV_H genes were identified by nucleotide sequencing (Sanger, F. *et al., Proc. Natl. Acad. Sci. USA* **74**:5463-5467 (1977)) and designated pAK2. Additional humanized variants were generated by site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The muMAb4D5 V_L and V_H gene segments in the transient expression vectors described above were then precisely replaced with their humanized versions.

Expression and Purification of MAb4D5 Variants. Appropriate MAb4D5 light and heavy chain cDNA expression vectors were co-transfected into an adenovirus transformed human

embryonic kidney cell line, 293 (Graham, F. L. *et al., J. Gen. Virol.* 36:59-72 (1977)) using a high efficiency procedure (Gorman, C. M. *et al., DNA & Prot. Engin. Tech.* 2:3-10 (1990); Gorman, C., in *DNA Cloning*, vol II, pp 143-190 (D. M. Glover, ed., IRL Press, Oxford, UK 1985)). Media were harvested daily for up to 5 days and the cells re-fed with serum free media. Antibodies were recovered from the media and affinity purified on protein A sepharose CL-4B (Pharmacia) as described by the manufacturer. The eluted antibody was buffer-exchanged into phosphate-buffered saline by G25 gel filtration, concentrated by ultrafiltration (Centriprep-30 or Centricon-100, Amicon), sterile-filtered (Millex-GV, Millipore) and stored at 4 °C. The concentration of antibody was determined by using both total immunoglobulin and antigen binding ELISAs. The standard used was huMAb4D5-5, whose concentration had been determined by amino acid composition analysis.

Cell Proliferation Assay. The effect of MAb4D5 variants upon proliferation of the human mammary adenocarcinoma cell line, SK-BR-3, was investigated as previously described (Fendly, B. M. *et al., Cancer Res.* 50:1550-1558 (1990)) using saturating MAb4D5 concentrations.

Affinity Measurements. The antigen binding affinity of MAb4D5 variants was determined using a secreted form of the $p185^{HER2}$ ECD prepared as described in Fendly, B. M. *et al., J. Biol. Resp. Mod.* 9:449-455 (1990). Briefly, antibody and $p185^{HER2}$ ECD were incubated in solution until equilibrium was found to be reached. The concentration of free antibody was then determined by ELISA using immobilized $p185^{HER2}$ ECD and used to calculate affinity (K_d) according to Friguet et al. (Friguet, B. *et al., J. Immunol. Methods* 77:305-319 (1985)).

RESULTS

Humanization of muMAb4D5. The muMAb4D5 V_L and V_H gene segments were first cloned by PCR and sequenced (Fig. 1). The variable genes were then simultaneously humanized by gene conversion mutagenesis using preassembled oligonucleotides (Fig. 2). A 311-mer oligonucleotide containing 39 mismatches to the template directed 24 simultaneous amino acid changes required to humanize muMAb4D5 V_L. Humanization of muMAb4D5 V_H required 32 amino acid changes which were installed with a 361-mer containing 59 mismatches to the muMAb4D5 template. Two out of 8 clones sequenced precisely encode huMAb4D5-5, although one of these clones contained a single nucleotide imperfection. The 6 other clones were essentially humanized but contained a small number of errors: < 3 nucleotide changes and < 1 single nucleotide deletion per kilobase. Additional humanized

variants (Table 3) were constructed by site-directed mutagenesis of huMAb4D5-5.

Expression levels of huMAb4D5 variants were in the range of 7 to 15 μg/ml as judged by ELISA using immobilized $p185^{HER2}$ ECD. Successive harvests of five 10 cm plates allowed 200 μg to 500 mg of each variant to be produced in a week. Antibodies affinity purified on protein A gave a single band on a Coomassie blue stained SDS polyacrylamide gel of mobility consistent with the expected M_r of ~ 150 kDa. Electrophoresis under reducing conditions gave 2 bands consistent with the expected M_r of free heavy (48 kDa) and light (23 kDa) chains (not shown). Amino terminal sequence analysis (10-cycles) gave the mixed sequence expected (see Fig. 1) from an equimolar combination of light and heavy chains (not shown).

huMAb4D5 Variants. In general, the FR residues were chosen from consensus human sequences (Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) and CDR residues from muMAb4D5. Additional variants were constructed by replacing selected human residues in huMAb4D5-1 with their muMAb4D5 counterparts. These are V_H residues 71, 73, 78, 93 plus 102 and V_L residues 55 plus 66 identified by our molecular modeling. V_H residue 71 has previously been proposed by others (Tramontano, A. *et al., J. Mol. Biol.* 215:175-182 (1990)) to be critical to the conformation of V_H-CDR2. Amino acid sequence differences between huMAb4D5 variant molecules are shown in Table 3, together with their $p185^{HER2}$ ECD binding affinity and maximal anti-proliferative activities against SK-BR-3 cells. Very similar K_d values were obtained for binding of MAb4D5 variants to either SK-BR-3 cells or to $p185^{HER2}$ ECD (Table 3). However, K_d estimates derived from binding of MAb4D5 variants to $p185^{HER2}$ ECD were more reproducible with smaller standard errors and consumed much smaller quantities of antibody than binding measurements with whole cells.

The most potent humanized variant designed by molecular modeling, huMAb4D5-8, contains 5 FR residues from muMAb4D5. This antibody binds the $p185^{HER2}$ ECD 3-fold *more* tightly than does muMAb4D5 itself (Table 3) and has comparable anti-proliferative activity with SK-BR-3 cells (Fig. 3). In contrast, huMAb4D5-1 is the most humanized but least potent muMAb4D5 variant, created by simply installing the muMAb4D5 CDRs into the consensus human sequences. huMAb4D5-1 binds the $p185^{HER2}$ ECD 80-fold *less* tightly than does the murine antibody and has no detectable anti-proliferative activity at the highest antibody concentration investigated (16 μg/ml).

The anti-proliferative activity of huMAb4D5 variants against $p185^{HER2}$ overexpressing SK-BR-3 cells is not simply correlated with their binding affinity for the $p185^{HER2}$ ECD. For

example, installation of three murine residues into the V_H domain of huMAb4D5-2 (D73T, L78A and A93S) to create huMAb4D5-3 does not change the antigen binding affinity but does confer significant anti-proliferative activity (Table 3).

The importance of V_H residue 71 (Tramontano, A. *et al.*, *J. Mol. Biol.* 215:175-182 (1990)) is supported by the observed 5-fold increase in affinity for $p185^{HER2}$ ECD on replacement of R71 in huMAb4D5-1 with the corresponding murine residue, alanine (huMAb4D5-2). In contrast, replacing V_H L78 in huMAb4D5-4 with the murine residue, alanine (huMAb4D5-5), does not significantly change the affinity for the $p185^{HER2}$ ECD or change anti-proliferative activity, suggesting that residue 78 is not of critical functional significance to huMAb4D5 and its ability to interact properly with the extracellular domain of $p185^{HER2}$.

V_L residue 66 is usually a glycine in human and murine κ chain sequences (Kabat, E. A. *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) but an arginine occupies this position in the muMAb4D5 k light chain. The side chain of residue 66 is likely to affect the conformation of V_L-CDR1 and V_L-CDR2 and the hairpin turn at 68-69 (Fig. 4). Consistent with the importance of this residue, the mutation V_L G66R (huMAb4D5-3 → huMAb4D5-5) increases the affinity for the $p185^{HER2}$ ECD by 4-fold with a concomitant increase in anti-proliferative activity.

From molecular modeling it appears that the tyrosyl side chain of muMAb4D5 V_L residue 55 may either stabilize the conformation of V_H-CDR3 or provide an interaction at the V_L-V_H interface. The latter function may be dependent upon the presence of V_H Y102. In the context of huMAb4D5-5 the mutations V_L E55Y (huMAb4D5-6) and V_H V102Y (huMAb4D5-7) individually increase the affinity for $p185^{HER2}$ ECD by 5-fold and 2-fold respectively, whereas together (huMAb4D5-8) they increase the affinity by 11-fold. This is consistent with either proposed role of V_L Y55 and V_H Y102.

Secondary Immune Function of huMAb4D5-8. MuMAb4D5 inhibits the growth of human breast tumor cells which overexpress $p185^{HER2}$ (Hudziak, R. M. *et al.*, *Molec. Cell. Biol.* 9:1165-1172 (1989)). The antibody, however, does not offer the possibility of direct tumor cytotoxic effects. This possibility does arise in huMAb4D5-8 as a result of its high affinity (K_d = 0.1 μM) and its human IgG_1 subtype. Table 4 compares the ADCC mediated by huMAb4D5-8 with muMAb4D5 on a normal lung epithelial cell line, WI-38, which expresses a low level of $p185^{HER2}$ and on SK-BR-3, which expresses a high level of $p185^{HER2}$. The results demonstrate that: (1) huMAb4D5 has a greatly enhanced ability to carry out ADCC as compared with its murine parent; and (2) that this activity may be selective for cell types

which overexpress $p185^{HER2}$.

DISCUSSION

MuMAb4D5 is potentially useful for human therapy since it is cytostatic towards human breast and ovarian tumor lines overexpressing the *HER2*-encoded $p185^{HER2}$ receptor-like tyrosine kinase. Since both breast and ovarian carcinomas are chronic diseases it is anticipated that the optimal MAb4D5 variant molecule for therapy will have low immunogenicity and will be cytotoxic rather than solely cytostatic in effect. Humanization of muMAb4D5 should accomplish these goals. We have identified 5 different huMAb4D5 variants which bind tightly to $p185^{HER2}$ ECD ($K_d \leq 1$ nM) and which have significant anti-proliferative activity (Table 3). Furthermore huMAb4D5-8 but not muMAb4D5 mediates ADCC against human tumor cell lines overexpressing $p185^{HER2}$ in the presence of human effector cells (Table 4) as anticipated for a human $\gamma 1$ isotype (Brüggemann, M. *et al., J. Exp. Med.* 166:1351-1361 (1987); Riechmann, L. *et al., Nature* 332:323-327 (1988)).

Rapid humanization of huMAb4D5 was facilitated by the gene conversion mutagenesis strategy developed here using long preassembled oligonucleotides. This method requires less than half the amount of synthetic DNA as does total gene synthesis and does not require convenient restriction sites in the target DNA. Our method appears to be simpler and more reliable than a variant protocol recently reported (Rostapshov, V. M. *et al., FEBS Lett.* 249:379-382 (1989)). Transient expression of huMAb4D5 in human embryonic kidney 293 cells permitted the isolation of a few hundred micrograms of huMAb4D5 variants for rapid characterization by growth inhibition and antigen binding affinity assays. Furthermore, different combinations of light and heavy chain were readily tested by co-transfection of corresponding cDNA expression vectors.

The crucial role of molecular modeling in the humanization of muMAb4D5 is illustrated by the designed variant huMAb4D5-8 which binds the $p185^{HER2}$ ECD 250-fold more tightly than the simple CDR loop swap variant, huMAb4D5-1. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. *et al., Nature* 332:323-327 (1988); Queen, C. *et al., Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989)). Here we have extended this earlier work by others with a designed humanized antibody which binds its antigen 3-fold *more* tightly than the parent rodent antibody. While this result is gratifying, assessment of the success of the molecular modeling must await the outcome of X-ray structure determination. From analysis of huMAb4D5 variants (Table 3) it is apparent that their anti-proliferative activity is not a

simple function of their binding affinity for $p185^{HER2}$ ECD. For example the huMAb4D5-8 variant binds $p185^{HER2}$ 3-fold more tightly than muMAb4D5 but the humanized variant is slightly less potent in blocking the proliferation of SK-BR-3 cells. Additional huMAb4D5 variants are currently being constructed in an attempt to identify residues triggering the anti-proliferative activity and in an attempt to enhance this activity.

In addition to retaining tight receptor binding and the ability to inhibit cell growth, the huMAb4D5-8 also confers a secondary immune function (ADCC). This allows for direct cytotoxic activity of the humanized molecule in the presence of human effector cells. The apparent selectivity of the cytotoxic activity for cell types which overexpress $p185^{HER2}$ allows for the evolution of a straightforward clinic approach to those human cancers characterized by overexpression of the HER2 protooncogene.

Table 3. $p185^{HER2}$ ECD binding affinity and anti-proliferative activities of MAb4D5 variants

	V_H Residue*					V_L Residue*			
MAb4D5 cell Variant proliferation‡	71 FR3	73 FR3	78 FR3	93 FR3	102 CDR3	55 CDR2	66 FR3	K_d† nM	Relative
huMAb4D5-1	R	D	L	A	V	E	G	25	102
huMAb4D5-2	Ala	D	L	A	V	E	G	4.7	101
huMAb4D5-3	Ala	Thr	Ala	Ser	V	E	G	4.4	66
huMAb4D5-4	Ala	Thr	L	Ser	V	E	Arg	0.82	56
huMAb4D5-5	Ala	Thr	Ala	Ser	V	E	Arg	1.1	48
huMAb4D5-6	Ala	Thr	Ala	Ser	V	Tyr	Arg	0.22	51
huMAb4D5-7	Ala	Thr	Ala	Ser	Tyr	E	Arg	0.62	53
huMAb4D5-8	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.10	54
muMAb4D5	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.30	37

* Human and murine residues are shown in one letter and three letter amino acid code respectively.

† K_d values for the $p185^{HER2}$ ECD were determined using the method of Friguet *et al.* (43) and the standard error of each estimate is ≤ ± 10%.

‡ Proliferation of SK-BR-3 cells incubated for 96 hr with MAb4D5 variants shown as a percentage of the untreated control as described (Hudziak, R. M. *et al.*, *Molec. Cell. Biol.* 9:1165-1172 (1989)). Data represent the maximal anti-proliferative effect for each variant (see Fig. 3A) calculated as the mean of triplicate determinations at a MAb4D5 concentration of 8 μg/ml. Data are all taken from the same experiment with an estimated standard error of

≤ ± 15%.

Table 4. Selectivity of antibody dependent tumor cell cytotoxicity mediated by huMAb4D5-8

		WI-38*		SK-BR-3	
Effector:Target ratio†		muMAb4D5	huMAb4D5-8	muMAb4D5	huMAb4D5-8
A.‡	25:1	<1.0	9.3	7.5	40.6
	12.5:1	<1.0	11.1	4.7	36.8
	6.25:1	<1.0	8.9	0.9	35.2
	3.13:1	<1.0	8.5	4.6	19.6
B.	25:1	<1.0	3.1	6.1	33.4
	12.5:1	<1.0	1.7	5.5	26.2
	6.25:1	1.3	2.2	2.0	21.0
	3.13:1	<1.0	0.8	2.4	13.4

* Sensitivity to ADCC of two human cell lines (WI-38, normal lung epithelium; and SK-BR-3, human breast tumor cell line) are compared. WI-38 expresses a low level of $p185^{HER2}$ (0.6 pg per μg cell protein) and SK-BR-3 expresses a high level of $p185^{HER2}$ (64 pg $p185^{HER2}$ per μg cell protein), as determined by ELISA (Fendly *et al., J. Biol. Resp. Mod.* 9:449-455 (1990)).

† ADCC assays were carried out as described in Brüggemann *et al., J. Exp. Med.* 166:1351-1361 (1987). Effector to target ratios were of IL-2 activated human peripheral blood lymphocytes to either WI-38 fibroblasts or SK-BR-3 tumor cells in 96-well microtiter plates for 4 hours at 37 °C. Values given represent percent specific cell lysis as determined by ^{51}Cr release. Estimated standard error in these quadruplicate determinations was ≤ ± 10%.

‡ Monoclonal antibody concentrations used were 0.1 μg/ml (A) and 0.1 μg/ml (B).

EXAMPLE 2. Schematic Method for Humanizing an Antibody Sequence

This example illustrates one stepwise elaboration of the methods for creating a humanized sequence described above. It will be understood that not all of these steps are essential to the claimed invention, and that steps may be taken in different order.

1. ascertain a consensus human variable domain amino acid sequence and prepare from it a consensus structural model.
2. prepare model of import (the non-human domain to be humanized) variable domain sequences and note structural differences with respect to consensus human model.
3. identify CDR sequences in human and in import, both by using Kabat (*supra*, 1987) and crystal structure criteria. If there is any difference in CDR identity from the different criteria, use of crystal structure definition of the CDR, but retain the Kabat residues as important framework residues to import.
4. substitute import CDR sequences for human CDR sequences to obtain initial "humanized" sequence.
5. compare import non-CDR variable domain sequence to the humanized sequence and note divergences.
6. Proceed through the following analysis for each amino acid residue where the import diverges from the humanized.
    a. If the humanized residue represents a residue which is generally highly conserved across all species, use the residue in the humanized sequence. If the residue is not conserved across all species, proceed with the analysis described in 6b.
    b. If the residue is not generally conserved across all species, ask if the residue is generally conserved in humans.
        i. If the residue is generally conserved in humans but the import residue differs, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological

activity of the CDRs by considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, leave the humanized residue unchanged.

ii. If the residue is also not generally conserved in humans, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs be considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, proceed to the next step.

a) Examine the structural models of the import and human sequences and determine if the residue is exposed on the surface of the domain or is buried within. If the residue is exposed, use the residue in the humanized sequence. If the residue is buried, proceed to the next step.

(i) Examine the structural models of the import and human sequences and determine if the residue is likely to affect the V_L - V_H interface. Residues involved with the interface include: 34L, 36L, 38L, 43L, 33L, 36L, 85L, 87L, 89L, 91L, 96L, 98L, 35H, 37H, 39H, 43H, 45H, 47H, 60H, 91H, 93H, 95H, 100H, and 103H. If no effect is likely, use the residue in the humanized sequence. If some affect is likely, substitute the import residue.

7. Search the import sequence, the consensus sequence and the humanized sequence for glycosylation sites outside the CDRs, and determine if this glycosylation site is likely to have any affect on

antigen binding and/or biological activity. If no effect is likely, use the human sequence at that site; if some affect is likely, eliminate the glycosylation site or use the import sequence at that site.

8. After completing the above analysis, determine the planned humanized sequence and prepare and test a sample. If the sample does not bind well to the target antigen, examine the particular residues listed below, regardless of the question of residue identity between the import and humanized residues.

   a. Examine particular peripheral (non-CDR) variable domain residues that may, due to their position, possibly interact directly with a macromolecular antigen, including the following residues (where the * indicates residues which have been found to interact with antigen based on crystal structures):

      i. Variable light domain: 36, 46, **49***, 63-70

      ii. Variable heavy domain: 2, **47***, 68, 70, 73-76.

   b. Examine particular variable domain residues which could interact with, or otherwise affect, the conformation of variable domain CDRs, including the following (not including CDR residues themselves, since it is assumed that, because the CDRs interact with one another, any residue in one CDR could potentially affect the conformation of another CDR residue) (L= LIGHT, H=HEAVY, residues appearing in bold are indicated to be structurally important according the Chothia *et al.*, Nature 342:877 (1989), and residues appearing in *italic* were altered during humanization by Queen *et al.* (PDL), Proc. Natl. Acad. Sci. USA 86:10029 (1989) and Proc. Natl. Acad. Sci. USA 88:2869 (1991).):

      i. Variable light domain:

         a) CDR-1 (residues 24L-34L): 2L, 4L, 66L-69L, **71L**

         b) CDR-2 (residues 50L-56L): 35L, 46L, *47L*, **48L**, *49L*, 58L, 62L, **64L**-66L, 71L, 73L

         c) CDR-3 (residues 89L-97L): 2L, 4L, 36L, 98L, 37H, 45H, 47H, 58H, 60H

      ii. Variable heavy domain:

a) CDR-1 (residues 26H-35H): 2H, 4H, 24H, 36H, 71H, 73H, 76H, 78H, 92H, *94H*

b) CDR-2 (residues 50H-55H): 49H, 69H, 69H, 71H, 73H, 78H

c) CDR-3 (residues 95H-102H): examine all residues as possible interaction partners with this loop, because this loop varies in size and conformation much more than the other CDRs.

9. If after step 8 the humanized variable domain still is lacking in desired binding, repeat step 8. In addition, re-investigate any buried residues which might affect the V_L - V_H interface (but which would not directly affect CDR conformation). Additionally, evaluate the accessibility of non-CDR residues to solvent.

EXAMPLE 3. Engineering a Humanized Bispecific F(ab')$_2$ Fragment

This example demonstrates the construction of a humanized bispecific antibody (BsF(ab')$_2$v1 by separate *E. coli* expression of each Fab' arm followed by directed chemical coupling *in vitro*. BsF(ab')$_2$ v1 (anti-CD3 / anti-p185^{HER2}) was demonstrated to retarget the cytotoxic activity of human CD3$^+$ CTL *in vitro* against the human breast tumor cell line, SK-BR-3, which overexpresses the p185^{HER2} product of the protooncogene *HER2*. This example demonstrates the minimalistic humanization strategy of installing as few murine residues as possible into a human antibody in order to recruit antigen-binding affinity and biological properties comparable to that of the murine parent antibody. This strategy proved very successful for the anti-p185^{HER2}arm of BsF(ab')$_2$v1. In contrast BsF(ab')$_2$ v1 binds to T cells *via* its anti-CD3 arm much less efficiently than does the chimeric BsF(ab')$_2$ which contains the variable domains of the murine parent anti-CD3 antibody. Here we have constructed additional BsF(ab')$_2$ fragments containing variant anti-CD3 arms with selected murine residues restored in an attempt to improve antibody binding to T cells. One such variant, Bs F(ab')$_2$ v9, was created by replacing six residues in the second hypervariable loop of the anti-CD3 heavy chain variable domain of BsF(ab')$_2$ v1 with their counterparts from the murine parent anti-CD3 antibody. BsF(ab')$_2$ v9 binds to T cells (Jurkat) much more efficiently than does BsF(ab')$_2$ v1 and almost as efficiently as the chimeric BsF(ab')$_2$. This improvement in the efficiency of T cell binding of the humanized BsF(ab')$_2$ is an important step in its development as a potential therapeutic agent for the treatment of p185^{HER2}-overexpressing cancers.

Bispecific antibodies (BsAbs) with specificities for tumor-associated antigens and surface markers on immune effector cells have proved effective for retargeting effector cells to kill tumor targets both *in vitro* and *in vivo* (reviewed by Fanger, M. W. *et al., Immunol. Today* 10: 92-99 (1989); Fanger, M. W. *et al., Immunol. Today* 12: 51-54 (1991); and Nelson, H., *Cancer Cells* 3: 163-172 (1991)). BsF(ab')$_2$ fragments have often been used in preference to intact BsAbs in retargeted cellular cytotoxicity to avoid the risk of killing innocent bystander cells binding to the Fc region of the antibody. An additional advantage of BsF(ab')$_2$ over intact BsAbs is that they

are generally much simpler to prepare free of contaminating monospecific molecules (reviewed by Songsivilai, S. and Lachmann, P. J., *Clin. Exp. Immunol.* **79**: 315-321 (1990) and Nolan, O. and O'Kennedy, R., *Biochim. Biophys. Acta* **1040**: 1-11 (1990)).

$BsF(ab')_2$ fragments are traditionally constructed by directed chemical coupling of Fab' fragments obtained by limited proteolysis plus mild reduction of the parent rodent monoclonal Ab (Brennan, M. *et al.*, *Science* **229**, 81-83 (1985) and *Glennie, M. J. et al., J. Immunol.* **139**: 2367-2375 (1987)). One such $BsF(ab')_2$ fragment (anti-glioma associated antigen / anti-CD3) was found to have clinical efficacy in glioma patients (Nitta, T. *et al., Lancet* **335**: 368-371 (1990) and another $BsF(ab')_2$ (anti-indium chelate / anti-carcinoembryonic antigen) allowed clinical imaging of colorectal carcinoma (Stickney, D. R. *et al., Antibody, Immunoconj. Radiopharm.* **2**: 1-13 (1989)). Future $BsF(ab')_2$ destined for clinical applications are likely to be constructed from antibodies which are either human or at least "humanized" (Riechmann, L. *et al., Nature* **332**: 323-327 (1988) to reduce their immunogenicity (Hale, G. *et al., Lancet* **i**: 1394-1399 (1988)).

Recently a facile route to a fully humanized $BsF(ab')_2$ fragment designed for tumor immunotherapy has been demonstrated (Shalaby, M. R. *et al., J. Exp. Med.* **175**: 217-225 (1992)). This approach involves separate *E. coli* expression of each Fab' arm followed by traditional directed chemical coupling *in vitro* to form the $BsF(ab')_2$. One arm of the $BsF(ab')_2$ was a humanized version (Carter, P. *et al., Proc. Natl. Acad. Sci. USA* (1992a) and Carter, P., *et al., Bio/Technology* **10**: 163-167 (1992b)) of the murine monoclonal Ab 4D5 which is directed against the $p185^{HER2}$ product of the protooncogene *HER2* (c-*erbB*-2) (Fendly, B. M. *et al.. Cancer Res.* **50**: 1550-1558 (1989)). The humanization of the antibody 4D5 is shown in Example 1 of this application. The second arm was a minimalistically humanized anti-CD3 antibody (Shalaby *et al. supra*) which was created by installing the CDR loops from the variable domains of the murine parent monoclonal Ab UCHT1 (Beverley, P. C. L. and Callard, R. E., *Eur. J. Immunol.* **11**: 329-334 (1981)) into the humanized anti-$p185^{HER2}$ antibody. The $BsF(ab')_2$ fragment containing the most potent humanized anti-CD3 variant (v1) was demonstrated by flow cytometry to bind specifically to a tumor target

overexpressing $p185^{HER2}$ and to human peripheral blood mononuclear cells carrying CD3. In addition, Bs F(ab')$_2$ v1 enhanced the cytotoxic effects of activated human CTL 4-fold against SK-BR-3 tumor cells overexpressing $p185^{HER2}$. The example descries efforts to improve the antigen binding affinity of the humanized anti-CD3 arm by the judicious recruitment of a small number of additional murine residues into the minimalistically humanized anti-CD3 variable domains.

MATERIALS AND METHODS

Construction of mutations in the anti-CD3 variable region genes.

The construction of genes encoding humanized anti-CD3 variant 1 (v1) variable light (V_L) and heavy (V_H) chain domains in phagemid pUC119 has been described (Shalaby *et al. supra*). Additional anti-CD3 variants were generated using an efficient site-directed mutagenesis method (Carter, P., *Mutagenesis: a practical approach*, (M. J. McPherson, Ed.), Chapter 1, IRL Press, Oxford, UK (1991)) using mismatched oligonucleotides which either install or remove unique restriction sites. Oligonucleotides used are listed below using lowercase to indicate the targeted mutations. Corresponding coding changes are denoted by the starting amino acid in one letter code followed by the residue numbered according to Kabat, E. A. *et al.*, *Sequences of Proteins of Immunological Interest*, 5th edition, National Institutes of Health, Bethesda, MD, USA (1991), then the replacement amino acid and finally the identity of the anti-CD3 variant:

HX11, 5' GTAGATAAATCCtctAACACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 11) V_H K75S, v6;

HX12, 5' GTAGATAAATCCAAAtctACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 12) V_H N76S, v7;

HX13, 5' GTAGATAAATCCtcttctACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 13) V_H K75S:N76S, v8;

X14, 5' CTTATAAAGGTGTTtCcACCTATaaCcAgAaatTCAA GGatCGTTTCACgATAtcCGTAGATAAATCC 3' (SEQ.ID.NO. 14) V_H T57S:A60N:D61Q:S62K:V63F:G65D, v9;

LX6, 5' CTATACCTCCCGTCTgcatTCTGGAGTCCC 3' (SEQ.ID. NO. 15)

V_L E55H, v11.

Oligonucleotides HX11, HX12 and HX13 each remove a site for BspMI, whereas LX6 removes a site for XhoI and HX14 installs a site for EcoRV (bold). Anti-CD3 variant v10 was constructed from v9 by site-directed mutagenesis using oligonucleotide HX13. Mutants were verified by dideoxynucleotide sequencing (Sanger, F. *et al.*, *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467 (1977)).

E. coli expression of Fab' fragments

The expression plasmid, pAK19, for the co-secretion of light chain and heavy chain Fd' fragment of the most preferred humanized anti-p185^{HER2} variant, HuMAb4D5-8, is described in Carter *et al.*, 1992b, *supra*. Briefly, the Fab' expression unit is bicistronic with both chains under the transcriptional control of the *pho*A promoter. Genes encoding humanized V_L and V_H domains are precisely fused on their 5' side to a gene segment encoding the heat-stable enterotoxin II signal sequence and on their 3' side to human k_1 C_L and IgG1 C_H1 constant domain genes, respectively. The C_H1 gene is immediately followed by a sequence encoding the hinge sequence CysAlaAla and followed by a bacteriophage λ t_0 transcriptional terminator. Fab' expression plasmids for chimeric and humanized anti-CD3 variants (v1 to v4, Shalaby *et al.*,*supra*; v6 to v12, this study) were created from pAK19 by precisely replacing anti-p185^{HER2} V_L and V_H gene segments with those encoding murine and corresponding humanized variants of the anti-CD3 antibody, respectively, by sub-cloning and site-directed mutagenesis. The Fab' expression plasmid for the most potent humanized anti-CD3 variant identified in this study (v9) is designated pAK22. The anti-p185^{HER2} Fab' fragment was secreted from *E. coli* K12 strain 25F2 containing plasmid pAK19 grown for 32 to 40 hr at 37°C in an aerated 10 liter fermentor. The final cell density was 120-150 OD_{550} and the titer of soluble and functional anti-p185^{HER2} Fab' was 1-2 g/liter as judged by antigen binding ELISA (Carter *et al.*, 1992b, *supra*). Anti-CD3 Fab' variants were secreted from *E. coli* containing corresponding expression plasmids using very similar fermentation protocols. The highest expression titers of chimeric and

humanized anti-CD3 variants were 200 mg/liter and 700 mg/liter, respectively, as judged by total immunoglobulin ELISA.

Construction of BsF(ab')$_2$ fragments

Fab' fragments were directly recovered from *E. coli* fermentation pastes in the free thiol form (Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter *et al.*, 1992b *supra*). Thioether linked BsF(ab')$_2$ fragments (anti-p185^{HER2} / anti-CD3) were constructed by the procedure of Glennie *et al. supra* with the following modifications. Anti-p185^{HER2} Fab'-SH in 100 mM Tris acetate, 5 mM EDTA (pH 5.0) was reacted with 0.1 vol of 40 mM N,N'-1,2-phenylenedimalemide (*o*-PDM) in dimethyl formamide for ~1.5 hr at 20 °C. Excess *o*-PDM was removed by protein G purification of the Fab' maleimide derivative (Fab'-mal) followed by buffer exchange into 20 mM sodium acetate, 5 mM EDTA (pH 5.3) (coupling buffer) using centriprep-30 concentrators (Amicon). The total concentration of Fab' variants was estimated from the measured absorbance at 280 nm (HuMAb4D5-8 Fab' $e^{0.1\%}$ = 1.56, Carter *et al.*, 1992b, *supra*). The free thiol content of Fab' preparations was estimated by reaction with 5, 5'-dithiobis(2-nitrobenzoic acid) as described by Creighton, T. E., *Protein structure: a practical approach*, (T. E. Creighton, Ed.), Chapter 7, IRL Press, Oxford, UK (1990). Equimolar amounts of anti-p185^{HER2} Fab'-mal (assuming quantitative reaction of Fab'-SH with *o*-PDM) and each anti-CD3 Fab'-SH variant were coupled together at a combined concentration of 1 to 2.5 mg/ml in the coupling buffer for 14 to 48 hr at 4 °C. The coupling reaction was adjusted to 4 mM cysteine at pH 7.0 and incubated for 15 min at 20 °C to reduce any unwanted disulfide-linked F(ab')$_2$ formed. These reduction conditions are sufficient to reduce inter-heavy chain disulfide bonds with virtually no reduction of the disulfide between light and heavy chains. Any free thiols generated were then blocked with 50 mM iodoacetamide. BsF(ab')$_2$ was isolated from the coupling reaction by S100-HR (Pharmacia) size exclusion chromatography (2.5 cm x 100 cm) in the presence of PBS. The BsF(ab')$_2$ samples were passed through a 0.2 mm filter flash frozen in liquid nitrogen and stored at -70 °C.

Flow cytometric analysis of F(ab')$_2$ binding to Jurkat cells

The Jurkat human acute T cell leukemia cell line was purchased from the American Type Culture Collection (Rockville, MD) (ATCC TIB 152) and grown as recommended by the ATCC. Aliquots of 10^6 Jurkat cells were incubated with appropriate concentrations of BsF(ab')$_2$ (anti-p185^{HER2} / anti-CD3 variant) or control mono-specific anti-p185^{HER2} F(ab')$_2$ in PBS plus 0.1% (w/v) bovine serum albumin and 10 mM sodium azide for 45 min at 4 °C. The cells were washed and then incubated with fluorescein-conjugated goat anti-human F(ab')$_2$ (Organon Teknika, West Chester, PA) for 45 min at 4 °C. Cells were washed and analyzed on a FACScan® (Becton Dickinson and Co., Mountain View, CA). Cells (8 x 10^3) were acquired by list mode and gated by forward light scatter *versus* side light scatter excluding dead cells and debris.

RESULTS

Design of humanized anti-CD3 variants

The most potent humanized anti-CD3 variant previously identified, v1, differs from the murine parent antibody, UCHT1 at 19 out of 107 amino acid residues within V_L and at 37 out of 122 positions within V_H (Shalaby *et al.,supra*) 1992). Here we recruited back additional murine residues into anti-CD3 v1 in an attempt to improve the binding affinity for CD3. The strategy chosen was a compromise between minimizing both the number of additional murine residues recruited and the number of anti-CD3 variants to be analyzed. We focused our attentions on a few CDR residues which were originally kept as human sequences in our minimalistic humanization regime. Thus human residues in V_H CDR2 of anti-CD3 v1 were replaced *en bloc* with their murine counterparts to give anti-CD3 v9: T57S:A60N:D61Q:S62K:V63F:G65D (Fig. 5). Similarly, the human residue E55 in V_L CDR2 of anti-CD3 v1 was replaced with histidine from the murine anti-CD3 antibody to generate anti-CD3 v11. In addition, V_H framework region (FR) residues 75 and 76 in anti-CD3 v1 were also replaced with their murine counterparts to create anti-CD3 v8: K75S:N76S. V_H residues 75 and 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might

influence antigen binding. Additional variants created by combining mutations at these three sites are described below.

Preparation of BsF(ab')$_2$ fragments

Soluble and functional anti-p185^{HER2} and anti-CD3 Fab' fragments were recovered directly from corresponding *E. coli* fermentation pastes with the single hinge cysteine predominantly in the free thiol form (75-100 % Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter *et al.*, 1992b, *supra*). Thioether-linked BsF(ab')$_2$ fragments were then constructed by directed coupling using *o*-PDM as described by Glennie *et al.*, *supra*. One arm was always the most potent humanized anti-p185^{HER2} variant, HuMAb4D5-8 (Carter *et al.*, 1992a, *supra*) and the other either a chimeric or humanized variant of the anti-CD3 antibody. Anti-p185^{HER2} Fab'-SH was reacted with *o*-PDM to form the maleimide derivative (Fab'-mal) and then coupled to the Fab'-SH for each anti-CD3 variant. F(ab')$_2$ was then purified away from unreacted Fab' by size exclusion chromatography as shown for a representative preparation (BsF(ab')$_2$ v8) in data not shown. The F(ab')$_2$ fragment represents ~54% of the total amount of antibody fragments (by mass) as judged by integration of the chromatograph peaks.

SDS-PAGE analysis of this BsF(ab')$_2$ v8 preparation under non-reducing conditions gave one major band with the expected mobility (M_r ~ 96 kD) as well as several very minor bands (data not shown). Amino-terminal sequence analysis of the major band after electroblotting on to polyvinylidene difluoride membrane Matsudaira, P., *J. Biol. Chem.* 262: 10035-10038 (1987) gave the expected mixed sequence from a stoichiometric 1:1 mixture of light and heavy chains (V_L / V_H: D/E, I/V, Q/Q, M/L, T/V, Q/E, S/S) expected for BsF(ab')$_2$. The amino terminal region of both light chains are identical as are both heavy chains and correspond to consensus human FR sequences. We have previously demonstrated that F(ab')$_2$ constructed by directed chemical coupling carry both anti-p185^{HER2} and anti-CD3 antigen specificities (Shalaby *et al.*, *supra*). The level of contamination of the BsF(ab')$_2$ with monospecific F(ab')$_2$ is likely to be very low since mock coupling reactions with either anti-p185^{HER2} Fab'-mal or anti-CD3 Fab'-SH alone did not yield detectable

quantities of F(ab')$_2$. Furthermore the coupling reaction was subjected to a mild reduction step followed by alkylation to remove trace amounts of disulfide-linked F(ab')$_2$ that might be present. SDS-PAGE of the purified F(ab')$_2$ under reducing conditions gave two major bands with electrophoretic mobility and amino terminal sequence anticipated for free light chain and thioether-linked heavy chain dimers.

Scanning LASER densitometry of a *o*-PDM coupled F(ab')$_2$ preparation suggest that the minor species together represent ~10% of the protein. These minor contaminants were characterized by amino terminal sequence analysis and were tentatively identified on the basis of stoichiometry of light and heavy chain sequences and their electrophoretic mobility (data not shown). These data are consistent with the minor contaminants including imperfect F(ab')$_2$ in which the disulfide bond between light and heavy chains is missing in one or both arms, trace amounts of Fab' and heavy chain thioether-linked to light chain.

Binding of BsF(ab')$_2$ to Jurkat cells

Binding of BsF(ab')$_2$ containing different anti-CD3 variants to Jurkat cells (human acute T cell leukemia) was investigated by flow cytometry (data not shown). BsF(ab')$_2$ v9 binds much more efficiently to Jurkat cells than does our starting molecule, BsF(ab')$_2$ v1, and almost as efficiently as the chimeric BsF(ab')$_2$. Installation of additional murine residues into anti-CD3 v9 to create v10 (V_H K75S:N76S) and v12 (V_H K75S:N76S plus V_L E55H) did not further improve binding of corresponding BsF(ab')$_2$ to Jurkat cells. Nor did recruitment of these murine residues into anti-CD3 v1 improve Jurkat binding: V_H K75S (v6), V_H N76S (v7), V_H K75S:N76S (v8), V_L E55H (v11) (not shown). BsF(ab')$_2$ v9 was chosen for future study since it is amongst the most efficient variants in binding to Jurkat cells and contains fewest murine residues in the humanized anti-CD3 arm. A monospecific anti-p185^{HER2} F(ab')$_2$ did not show significant binding to Jurkat cells consistent with the interaction being mediated through the anti-CD3 arm.

DISCUSSION

A minimalistic strategy was chosen to humanize the anti-p185^{HER2}

(Carter *et al.*, 1992a, *supra*) and anti-CD3 arms (Shalaby *et al.*, *supra*) of the $BsF(ab')_2$ in this study in an attempt to minimize the potential immunogenicity of the resulting humanized antibody in the clinic. Thus we tried to install the minimum number of murine CDR and FR residues into the context of consensus human variable domain sequences as required to recruit antigen-binding affinity and biological properties comparable to the murine parent antibody. Molecular modeling was used firstly to predict the murine FR residues which might be important to antigen binding and secondly to predict the murine CDR residues that might *not* be required. A small number of humanized variants were then constructed to test these predictions.

Our humanization strategy was very successful for the anti-$p185^{HER2}$ antibody where one out of eight humanized variants (HuMAb4D5-8, IgG1) was identified that bound the $p185^{HER2}$ antigen ~3-fold more tightly than the parent murine antibody (Carter *et al.*, 1992a, *supra*). HuMAb4D5-8 contains a total of five murine FR residues and nine murine CDR residues, including V_H CDR2 residues 60-65, were discarded in favor of human counterparts. In contrast, $BsF(ab')_2$ v1 containing the most potent humanized anti-CD3 variant out of four originally constructed (Shalaby *et al.*, *supra*) binds J6 cells with an affinity (K_d) of 140 nM which is ~70-fold weaker than that of the corresponding chimeric $BsF(ab')_2$.

Here we have restored T cell binding of the humanized anti-CD3 close to that of the chimeric variant by replacing six human residues in V_H CDR2 with their murine counterparts: T57S:A60N:D61Q:S62K:V63F:G65D (anti-CD3 v9, Fig. 5). It appears more likely that these murine residues enhance antigen binding *indirectly* by influencing the conformation of residues in the N-terminal part of V_H CDR2 rather than by *directly* contacting antigen. Firstly, only N-terminal residues in V_H CDR2 (50-58) have been found to contact antigen in one or more of eight crystallographic structures of antibody/antigen complexes (Kabat *et al.*, *supra*; and Mian, I. S. *et al.*, *J. Mol. Biol.* 217: 133-151 (1991), Fig. 5). Secondly, molecular modeling suggests that residues in the C-terminal part of V_H CDR2 are at least partially buried (Fig. 5). $BsF(ab')_2$ v9 binds to SK-BR-3 breast tumor cells with equal efficiency to $BsF(ab')_2$ v1 and chimeric $BsF(ab')_2$ as anticipated since the anti-$p185^{HER2}$ arm is identical in all of these molecules (Shalaby *et al.*, *supra*, not

shown).

Our novel approach to the construction of BsF(ab')$_2$ fragments exploits an *E. coli* expression system which secretes humanized Fab' fragments at gram per liter titers and permits their direct recovery as Fab'-SH (Carter *et al.*, 1992b, *supra*). Traditional directed chemical coupling of Fab'-SH fragments is then used to form BsF(ab')$_2$ *in vitro* (Brennan *et al.*,*supra*; and Glennie *et al.*, *supra*). This route to Fab'-SH obviates problems which are inherent in their generation from intact antibodies: differences in susceptibility to proteolysis and nonspecific cleavage resulting in heterogeneity, low yield as well as partial reduction that is not completely selective for the hinge disulfide bonds. The strategy of using *E. coli*-derived Fab'-SH containing a single hinge cysteine abolishes some sources of heterogeneity in BsF(ab')$_2$ preparation such as intra-hinge disulfide formation and contamination with intact parent antibody whilst greatly diminishes others, eg. formation of F(ab')$_3$ fragments.

BsF(ab')2 fragments constructed here were thioether-linked as originally described by Glennie *et al.*, *supra* with future *in vivo* testing of these molecules in mind. Thioether bonds, unlike disulfide bonds, are not susceptible to cleavage by trace amounts of thiol, which led to the proposal that thioether-linked F(ab')$_2$ may be more stable than disulfide-linked F(ab')$_2$ *in vivo* (Glennie *et al.*, *supra*). This hypothesis is supported by our preliminary pharmacokinetic experiments in normal mice which suggest that thioether-linked BsF(ab')$_2$ v1 has a 3- fold longer plasma residence time than BsF(ab')$_2$ v1 linked by a single disulfide bond. Disulfide and thioether-linked chimeric BsF(ab')$_2$ were found to be indistinguishable in their efficiency of cell binding and in their retargeting of CTL cytotoxicity, which suggests that *o*-PDM directed coupling does not compromise binding of the BsF(ab')$_2$ to either antigen (not shown). Nevertheless the nature of the linkage appears not to be critical since a disulfide-linked BsF(ab')$_2$ (murine anti-p185^{HER2} / murine anti-CD3) was recently shown by others (Nishimura *et al.*, *Int. J. Cancer* 50: 800-804 (1992) to have potent anti-tumor activity in nude mice. Our previous study (Shalaby *et al.*, *supra*) together with this one and that of Nishimura, T. *et al.*, *supra* improve the potential for using BsF(ab')$_2$ in targeted immunotherapy of p185^{HER2}-overexpressing cancers in humans.

EXAMPLE 4. Humanization of an anti-CD18 antibody

A murine antibody directed against the leukocyte adhesion receptor β-chain (known as the H52 antibody) was humanized following the methods described above. Figures 6A and 6B provide amino acid sequence comparisons for the murine and humanized antibody light chains and heavy chains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: Immunoglobulin Variants

(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) APPLICATION DATE: 14-JUN-1991

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Adler, Carolyn R.
(B) REGISTRATION NUMBER: 32,324
(C) REFERENCE/DOCKET NUMBER: 709P1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/225-2614
(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

:p 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 CCXXCXCCGA 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:

AGGTXXAXCT GCAGXAGTCX 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:

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

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

Glu Tyr Thr Met His Trp Met Lys Gln Ser His Gly Lys Ser Leu
        35                  40                  45

Glu Trp Ile Gly Gly Phe Asn Pro Lys Asn Gly Gly Ser Ser His
    50                  55                  60

Asn Gln Arg Phe Met Asp Lys Ala Thr Leu Ala Val Asp Lys Ser
65                  70                  75

Thr Ser Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu Asp
            80                  85                  90

Ser Gly Ile Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
        95                  100                 105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val
    110                 115                 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
125                 130                 135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
            140                 145                 150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
        155                 160                 165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
    170                 175                 180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
185                 190                 195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
            200                 205                 210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
        215                 220                 225
```

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
230 235 240

Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
245 250 255

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
260 265 270

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
275 280 285

Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
290 295 300

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
305 310 315

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
320 325 330

Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
335 340 345

Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
350 355 360

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
365 370 375

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
380 385 390

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
395 400 405

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
410 415 420

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
425 430 435

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
440 445 450

Ser Pro Gly Lys
454

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 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
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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

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, 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, 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, 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 substituting an amino acid residue for the human residue at a site 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.

11. The humanized antibody variable domain of claim 10, wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR was obtained.

12. The humanized antibody variable domain of claim 10, wherein no human FR residue other than those set forth in the group has been substituted.

13. A polypeptide comprising the amino acid sequence:
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLI
YSASFLESGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTF
GQGTKVEIKRT

14. A polypeptide comprising the sequence:
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWV
ARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC
SRWGGDGFYAMDVWGQGTLVTVSS

15. A method for engineering a humanized antibody comprising introducing amino acid residues from an import antibody variable domain into an amino acid sequence representing a consensus of mammalian antibody variable domain sequences.

16. A computer comprising the sequence data of the following amino acid sequence:
    a. DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKA
    PKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
    QYNSLPYTFGQGTKVEIKRT, or
    b. EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGK
    GLEWVAVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLR
    AEDTAVYYCSRWGGDGFYAMDVWGQGTLVTVSS

17. A computer representation of the following amino acid sequence:
    a. DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKA
    PKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
    QYNSLPYTFGQGTKVEIKRT, or
    b. EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGK
    GLEWVAVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLR
    AEDTAVYYCSRWGGDGFYAMDVWGQGTLVTVSS

18. A method comprising storing a computer representation of the following amino acid sequence:

a. DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKA
PKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
QYNSLPYTFGQGTKVEIKRT, or

b. EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGK
GLEWVAVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLR
AEDTAVYYCSRWGGDGFYAMDVWGQGTLVTVSS

INTERNATIONAL SEARCH REPORT

International Application No CT/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[10]	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

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 OCTOBER 1992	02. 11. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	NAUCHE S.A.

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

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

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
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 SEARCH REPORT

Interr tnal application No.
PCT/US92/05126

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. [X] 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.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 93/07832

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
WO-A-9222653	23-12-92	AU-A-	2250992	12-01-93

Form PCT/ISA/210 (patent family annex) (July 1992)

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
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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

FIGURE 1A: V_L DOMAIN

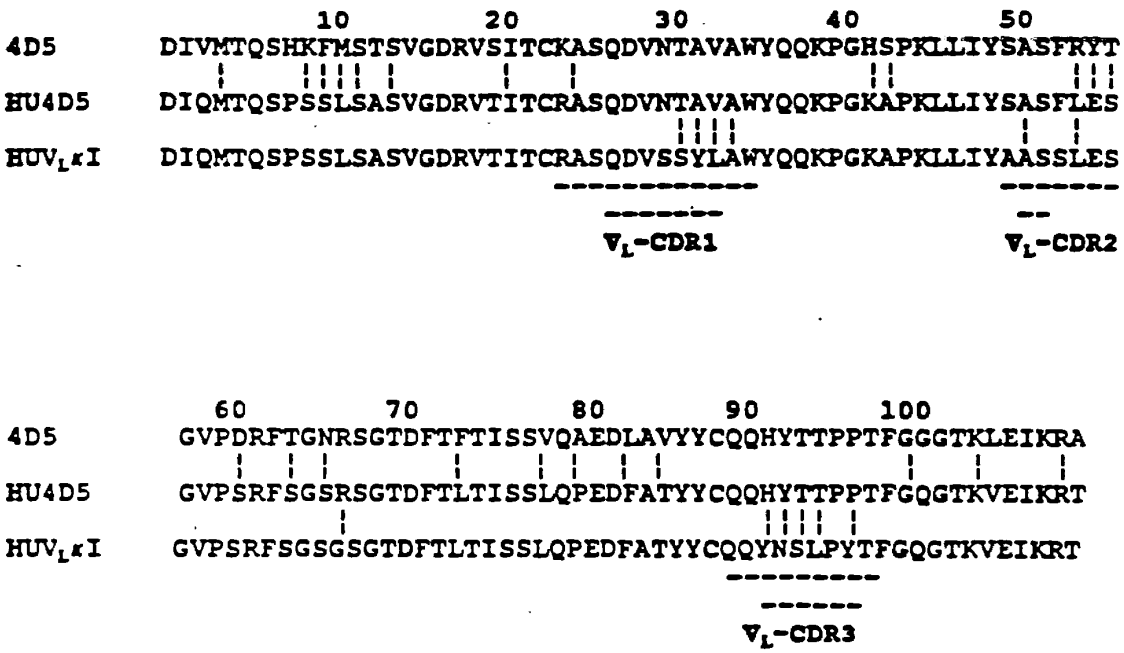


FIGURE 1B: V_H DOMAIN

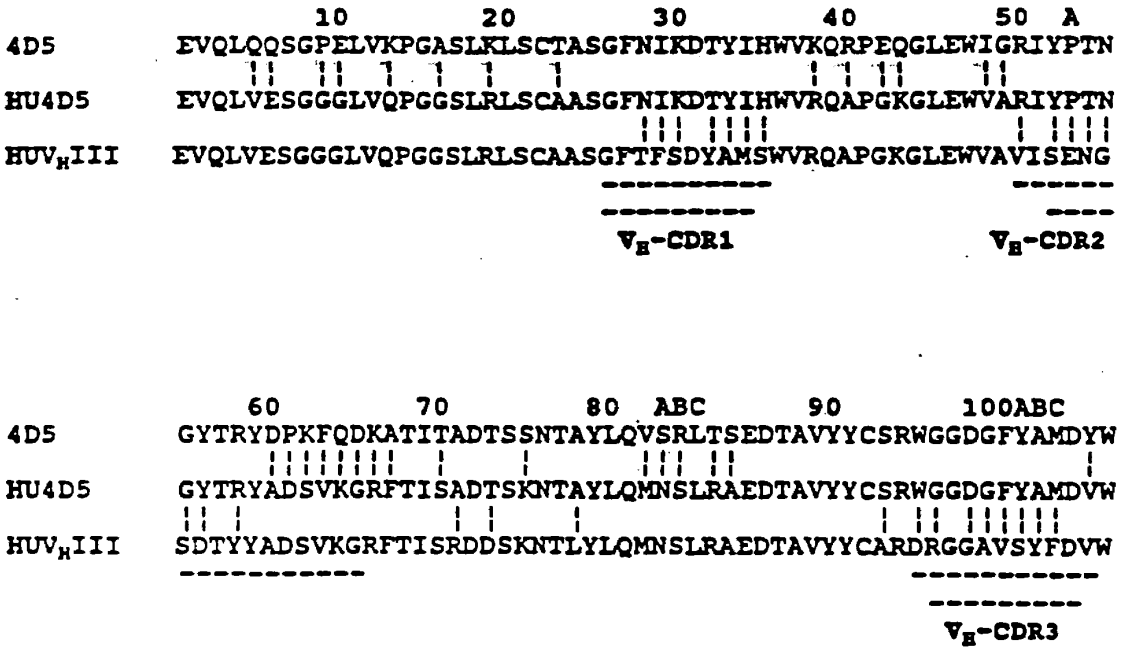
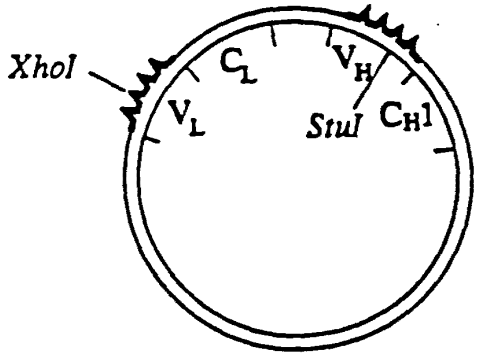


FIGURE 2

Anneal huV_L or huV_H oligomers to pAK1 template

1. Ligate
2. Isolate assembled oligomers
3. Anneal to pAK1 template (*XhoI*$^-$, *StuI*$^+$)
4. Extend and ligate



1. Transform *E. coli*
2. Isolate phagemid pool
3. Enrich for huV_L and huV_H(*Xho I*$^+$, *StuI*$^-$)
4. Sequence verify

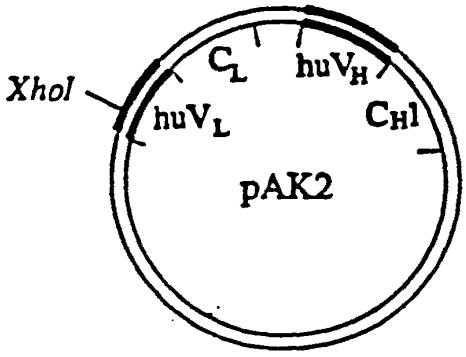


FIGURE 3

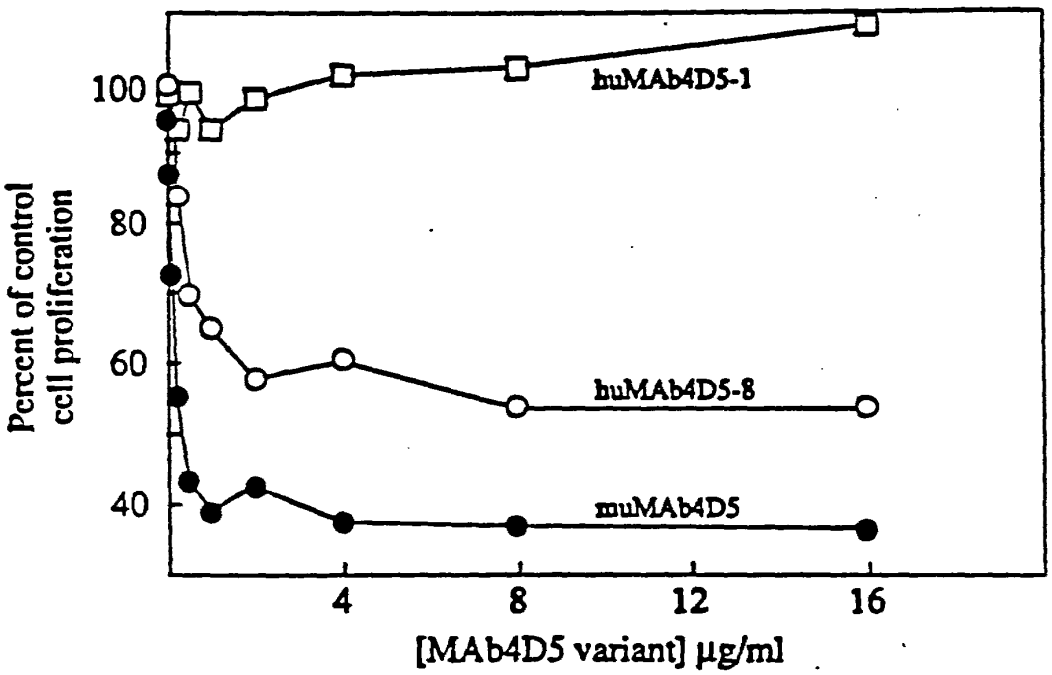
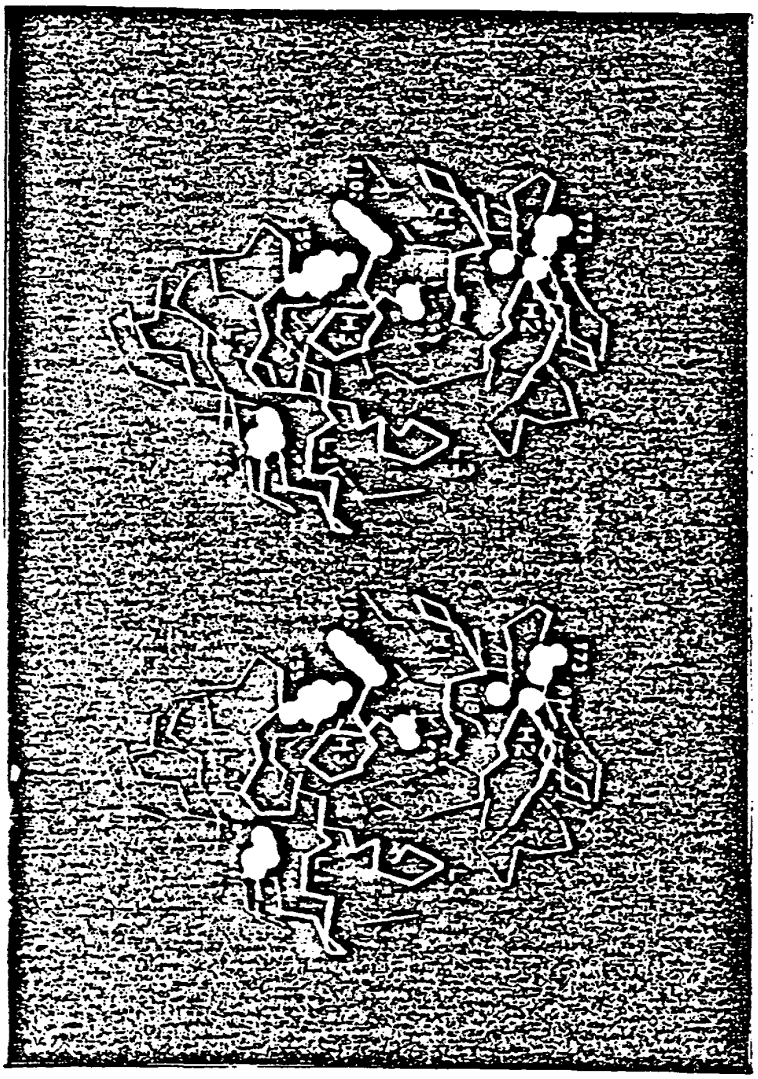

huMAb4D5-1
huMAb4D5-8
muMAb4D5
Percent of control cell proliferation
100
80
60
40
4
8
12
16
[MAb4D5 variant] µg/ml

FIGURE 4



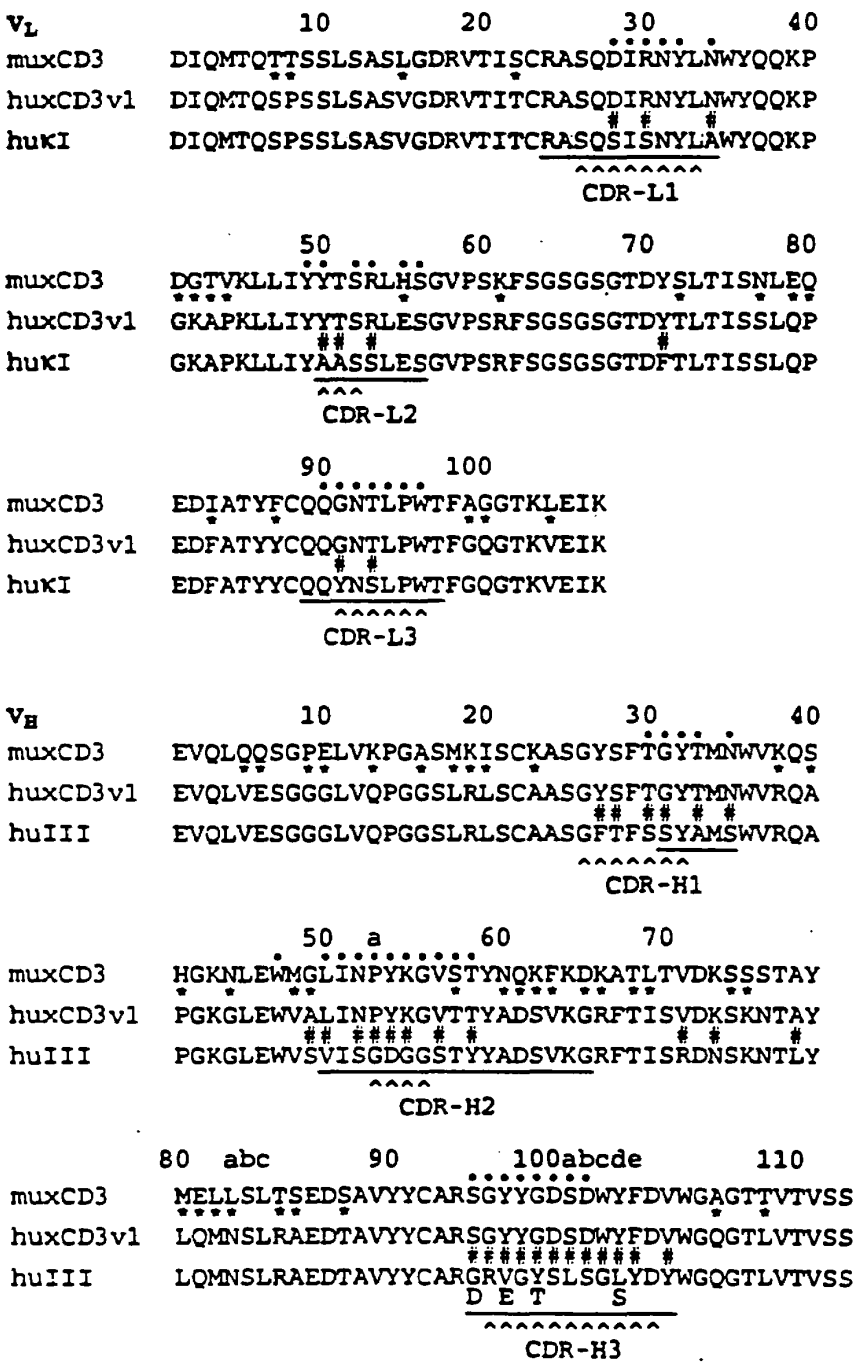
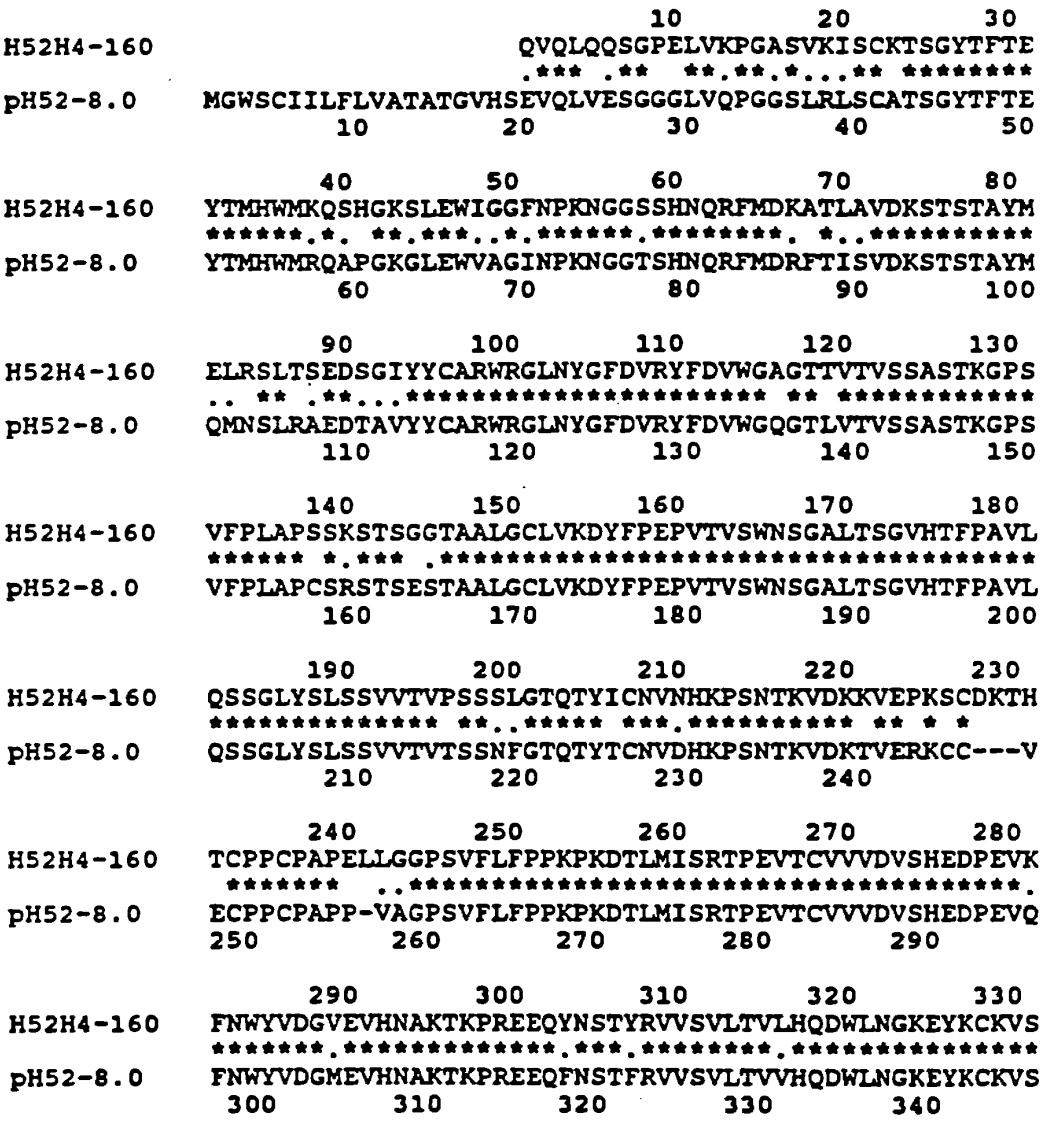

VL
10 20 30 40
muxCD3 DIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKP
huxCD3v1 DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKP
huκI DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKP
CDR-L1
50 60 70 80
muxCD3 DGTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQ
huxCD3v1 GKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISSLQP
huκI GKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQP
CDR-L2
90 100
muxCD3 EDIATYFCQQGNTLPWTFAGGTKLEIK
huxCD3v1 EDFATYYCQQGNTLPWTFGQGTKVEIK
huκI EDFATYYCQQYNSLPWTFGQGTKVEIK
CDR-L3
VH
10 20 30 40
muxCD3 EVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQS
huxCD3v1 EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQA
huIII EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA
CDR-H1
50 a 60 70
muxCD3 HGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAY
huxCD3v1 PGKGLEWVALINPYKGVTTYADSVKGRFTISVDKSKNTAY
huIII PGKGLEWVSVISGDGGSTYYADSVKGRFTISRDNSKNTLY
CDR-H2
80 abc 90 100abcde 110
muxCD3 MELLSLTSEDSAVYYCARSGYYGDSDWYFDVWGAGTTVTVSS
huxCD3v1 LQMNSLRAEDTAVYYCARSGYYGDSDWYFDVWGQGTLVTVSS
huIII LQMNSLRAEDTAVYYCARGRVGYSLSGLYDYWGQGTLVTVSS
D E T S
CDR-H3

FIGURE 5

FIGURE 6A


H52H4-160 QVQLQQSGPELVKPGASVKISCKTSGYTFTE
pH52-8.0 MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCATSGYTFTE
H52H4-160 YTMHWMKQSHGKSLEWIGGFNPKNGGSSHNQRFMDKATLAVDKSTSTAYM
pH52-8.0 YTMHWMRQAPGKGLEWVAGINPKNGGTSHNQRFMDRFTISVDKSTSTAYM
H52H4-160 ELRSLTSEDSGIYYCARWRGLNYGFDVRYFDVWGAGTTVTVSSASTKGPS
pH52-8.0 QMNSLRAEDTAVYYCARWRGLNYGFDVRYFDVWGQGTLVTVSSASTKGPS
H52H4-160 VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
pH52-8.0 VFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
H52H4-160 QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
pH52-8.0 QSSGLYSLSSVVTVTSSNFGTQTYTCNVDHKPSNTKVDKTVERKCC---V
H52H4-160 TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
pH52-8.0 ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQ
H52H4-160 FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
pH52-8.0 FNWYVDGMEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVS

```
                      340       350       360       370       380
H52H4-160     NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
              **.***********.****************************************
pH52-8.0      NKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
               350       360       370       380       390

                      390       400       410       420       430
H52H4-160     SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
              **********************.*****************************
pH52-8.0      SDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFS
               400       410       420       430       440

                      440       450
H52H4-160     CSVMHEALHNHYTQKSLSLSPGK
              ************************
pH52-8.0      CSVMHEALHNHYTQKSLSLSPGK
               450       460
```

FIGURE 6B

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

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

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

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

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

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(71) Applicant *(for all designated States except US)*: THE WELLCOME FOUNDATION LIMITED [GB/GB]; Unicorn House, 160 Euston Road, London NW1 2BP (GB).

(71)(72) Applicant and Inventor: WALDMANN, Herman [GB/GB]; University of Cambridge, Department of Pathology, Tennis Court Road, Cambridge, Cambridgeshire CB2 1QP (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : SIMS, Martin [GB/GB]; The Wellcome Foundation Limited, Langley Court, Beckenham, Kent BR3 3BS (GB). CROWE, Scott [GB/GB]; Wellcome Foundation Limited, Langley Court, Beckenham, Kent BR3 3BS (GB).

(74) Agent: STOTT, M., J.; The Wellcome Foundation Limited, Langley Court, Beckenham, Kent BR3 3BS (GB).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: HUMANIZED ANTIBODY AGAINST CD18

(57) Abstract

A humanized antibody having all or part of the CDRs as defined and capable of binding to the human CD-18 antigen. The antibody is of use in therapy in treating leukocyte mediated conditions such as inhibiting ingress of leukocytes into the lung and other organs and treatment of inflammation.

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HUMANIZED ANTIBODY AGAINST CD18

The present invention relates to an antibody which binds to the CD18 antigen, to the preparation of such an antibody and to a pharmaceutical composition which contains the antibody.

Antibodies typically comprise two heavy chains linked together by disulphide bonds and two light chains. Each light chain is linked to a respective heavy chain by disulphide bonds. 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 light chain variable domain is aligned with the variable domain of the heavy chain. The light chain constant domain is 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 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 a framework of four regions, whose sequences are relatively conserved, connected by three complementarity determining regions (CDRs). The four framework regions largely adopt a beta-sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the beta-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. CDRs and framework regions of antibodies may be determined by reference to Kabat et al ("Sequences of proteins of immunological interest" US Dept. of Health and Human Services, US Government Printing Office, 1987).

The preparation of an altered antibody in which the CDRs are derived from a different species than the framework of the antibody's variable domains is disclosed

in EP-A-0239400. The CDRs may be derived from a rat or mouse monoclonal antibody. The framework of the variable domains, and the constant domains, of the altered antibody may be derived from a human antibody. Such a humanised antibody elicits a negligible immune response when administered to a human compared to the immune response mounted by a human against a rat or mouse antibody. Humanised CAMPATH-1 antibody is disclosed in EP-A-0328404 (Campath is a Trade Mark of The Wellcome group of companies).

According to one aspect of the present invention, there is provided a humanised antibody in which sufficient of the amino acid sequence of each CDR shown below is provided such that the antibody is capable of binding to the human CD18 antigen:

light chain: CDR1 (SEQ ID NOS: 3 and 4)
CDR2 (SEQ ID NOS: 5 and 6)
CDR3 (SEQ ID NOS: 7 and 8)
heavy chain: CDR1 (SEQ ID NOS: 11 and 12)
CDR2 (SEQ ID NOS: 13 and 14)
CDR3 (SEQ ID NOS: 15 and 16)

According to another aspect the invention provides a DNA molecule encoding a humanised antibody in which sufficient of the amino acid sequence of each CDR shown above is provided such that the antibody is capable of binding to the human CD-18 antigen.

The antibody preferably has the structure of a natural antibody or a fragment thereof. The antibody may therefore comprise a complete antibody, a $(Fab')_2$ fragment, a Fab fragment, a light chain dimer or a heavy chain dimer. The antibody may be an IgG such as IgG1, IgG2, IgG3 or IgG4; or IgM, IgA, IgE or IgD. The constant domain of the antibody heavy chain may be selected accordingly. The light chain constant domain may be a kappa or lambda constant domain.

The antibody may be a chimeric antibody of the type

described in WO 86/01533. A chimeric antibody according to WO 86/01533 comprises an antigen binding region and a non-immunoglobulin region. The antigen binding region is an antibody light chain variable domain and/or heavy chain variable domain. Typically the chimeric antibody comprises both light and heavy chain variable domains. The non-immunoglobulin region is fused to the C-terminus of the antigen binding region. The non-immunoglobulin region is typically a non-immunoglobulin protein and may be an enzyme region, a region derived from a protein having known binding specificity, from a protein toxin or indeed from any protein expressed by a gene. The non-immunoglobulin region may be a carbohydrate region. The two regions of the chimeric antibody may be connected via a cleavable linker sequence.

The light chain CDRs 1 to 3 and heavy chain CDRs 1 to 3 of SEQ ID NOS: 3 to 8 and SEQ ID NOS: 11 to 16 respectively are the CDRs of the YFC51.1.1 rat antibody which is a CD18 antibody. The specificity of a humanised antibody for the human CD18 antigen can be determined by flow cytometry, monocyte adhesion and/or by T-cell proliferation assays as follows:

Monocyte (MNC) Adhesion

MNC's are treated with the phorbol diester PDBu (10^{-9} M) in the presence and absence of antibody (20μl) for 5 minutes. These cells are then transferred to bovine aortic endothelial cell (BAEC) monolayers and incubated for 30 minutes in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Non-adherent cells are removed by washing in phosphate buffered saline (PBS) three times. The adherent cells are then lysed *in situ* with 50μl, 0.5% hexadecyltrimethyl ammonium bromide. Dianisidine dihydrochloride (0.63mM) containing 0.4mM hydrogen peroxide is added (250μl) to each well and incubated for a further 10 minutes. Enzyme activity is then assessed using the presence of monocyte-specific myeloperoxidase, recorded as

an increase in absorbance. The optical density of the samples can then be recorded at 450nm using a multi-well plate reader (Anthos series, Lab Teck instruments). Comparisons can then be made between treated and untreated samples (Bath et al, J. Immunol. Meth., 118, 59-65, (1989)).

Flow cytometry

Surface labelling of rat, rabbit, guinea-pig and human monocytes with antibody is carried out according to the method of Gladwin et al., (Biochim. Biophys. Acta., 1052, 166-172 (1990)). Briefly, 1 ml aliquots of cells suspension ($5x10^6$) are incubated with the appropriate antibody, monodispersed and incubated on melting ice for 30 minutes. The cells are twice washed in PBS and incubated for a further 30 minutes with a 1:200 dilution of rabbit anti-rat $F(ab')_2$ FITC conjugate on melting ice. The cells are finally washed three times in PBS and fixed in 0.1% para-formaldehyde. Analysis of surface labelling can be performed using an Epics Elite flow cytometer (Coulter cytometry, Hialhea, FL) using standard computer, electronics and optics. The Elite is configured with a 15mW 488nm Argon-ion laser (Cyonics model 2201, San Jose, CA). Monocyte and lymphocyte populations are separated by forward angle light scatter and side scatter. Green fluorescence data for 2×10^4 monocytes is collected using bit-map gating and collected on a three decade log scale. Green fluorescence data for 2×10^4 neutrophils is collected in a similar manner. For each sample, mean fluorescence intensity in the presence of the primary mAb is compared with cells incubated with rabbit anti-rat $F(ab')_2$ FITC fragments alone and the percentage labelling of the cells determined. Samples can be labelled in triplicate and repeat experiments can be performed on three separate occasions.

T-cell proliferation assay

Human mononuclear cells are prepared from defibrinated blood using density gradient separation over Ficoll-paque. Lymphocytes (2 x 10^5 cells) are cultured in each well of a flat bottomed 96-well microtitre plate (Nunclon, Roskild, Denmark), in RPMI 1640 supplemented with 10% autologous serum, 2mM glutamine and 100iU penicillin/ 100μg ml^{-1} streptomycin. Triplicate cultures are set up with the medium alone or with antigen (Tetanus Toxoid, 3μg ml^{-1}) or mitogen (PHA, 1μg ml^{-1}), in the presence or absence of different concentrations of monoclonal antibodies. Cells are cultured at 37°C in a humidified atmosphere of 95% air, 5% CO_2 for five days. Wells are then pulsed with 1μCi [methyl^{3}H] thymidine (2Ci $mmol^{-1}$, Amersham), harvested 18 hours later and radioactivity counted by liquid scintillation using a B counter (LKB, Betaplate, Sweden). The results are expressed as mean +/- SEM.

Suitably, the CDRs of a humanised antibody are the light chain CDRs 1 to 3 and the heavy chain CDRs 1 to 3 above. The amino acid sequences of these CDRs may be changed, however. The amino acid sequence of each CDR may be changed by up to 40% by amino acid substitutions, insertions and/or deletions, for example by up to 30%, up to 20% or up to 10%.

Each CDR may therefore include one or two amino acid substitutions, insertions and/or deletions. There may be up to three amino acid substitutions, insertions, and/or deletions in light chain CDR. Up to four amino acid substitutions, insertions and/or deletions may be present in light chain CDR1 or heavy chain CDR3. Up to six amino acid substitutions, insertions and/or deletions may be present in heavy chain CDR2. Preferably the amino acid sequence of each CDR is substantially homologous to that of each CDR of YFC 51.1.1.

The framework and the constant domains of the antibody are human framework and human constant domains.

Preferably the framework of the variable region of the antibody heavy chain is substantially homologous to the corresponding framework of the human protein NEWM (Saul et al, J. Biol. Chem. 25, 585-597, (1987)). Homology in respect of the framework is generally 80% or more with respect to NEWM, for example 90% or more or 95% or more. A number of amino acid substitutions, insertions and/or deletions may be present. Candidate framework changes that may be made to restore binding include changes of amino acid residues 27, 30, 48, 66, 67, 71, 91, 93 and 94. The amino acid numbering is according to Kabat et al.

The framework of the variable region of the antibody light chain is typically substantially homologous to the variable domain framework of the protein REI (Epp et al, Eur. J. Biochem. 45, 513-524, (1974)). Homology in respect of the framework is generally 80% or more with respect to REI, for example 90% or more or 95% or more. A number of amino acid substitutions, insertions and/or deletions may be present, for example at amino acid residue 71 according to the numbering of Kabat et al.

A humanised antibody is prepared according to the invention by a process which comprises maintaining a host transformed with a first expression vector which encodes the light chain of the humanised antibody and with a second expression vector which encodes the heavy chain of the humanised antibody under such conditions that each chain is expressed and isolating the humanised antibody formed by assembly of the thus-expressed chains.

The first and second expression vectors may be the same vector. The invention further provides:

- a DNA sequence encoding the light chain or the heavy chain of the humanised antibody;
- an expression vector which incorporates a said DNA sequence; and
- a host transformed with a said expression vector.

Each chain of the antibody may be prepared by CDR

replacement. The CDRs of a variable region of a light or heavy chain of a human antibody are replaced by sufficient of the amino acid sequence of each CDR of the rat anti-human-CD18 antibody YFC51.1.1 such that the resulting antibody is capable of binding to the CD18 antigen. The CDR-encoding regions of DNA encoding a hypervariable region of a human antibody chain are replaced by DNA encoding the desired CDRs. If appropriate, this altered DNA is linked to DNA encoding a constant domain for the antibody chain. The DNA is cloned into an expression vector. The expression vector is introduced into a compatible host cell which is cultured under such conditions that the antibody chain is expressed. Complementary antibody chains which are co-expressed in this way may then assemble to form the humanised antibody.

The present invention is described herein with particular reference to the production of a humanised antibody having CDRs derived directly or indirectly from the rat antibody YFC51.1.1. However the techniques described herein can equally be used to derive other humanised anti CD-18 antibodies. According to a further aspect, the present invention provides a humanised (CDR grafted) anti CD-18 antibody.

There are four general steps to humanise a monoclonal antibody. These are:

(1) determining the nucleotide and predicted amino acid sequence of the starting antibody light and heavy variable domains;

(2) designing the humanised antibody, i.e. deciding which antibody framework region to use during the humanising process;

(3) the actual humanising methodologies/techniques; and

(4) the transfection and expression of the humanised antibody.

Step 1: Determining the nucleotide and predicted amino acid sequence of the antibody light and heavy chain variable domains

To humanise an antibody only the amino acid sequence of antibody's heavy and light chain variable domains needs to be known. The sequence of the constant domains is irrelevant because these do not contribute to the reshaping strategy. The simplest method of determining an antibody's variable domain amino acid sequence is from cloned cDNA encoding the heavy and light chain variable domain.

There are two general methods for cloning a given antibody's heavy and light chain variable domain cDNAs: (1) via a conventional cDNA library, or (2) via the polymerase chain reaction (PCR). Both of these methods are widely known. Given the nucleotide sequence of the cDNAs, it is a simple matter to translate this information into the predicted amino acid sequence of the antibody variable domains. In the present instance, the nucleotide sequence and predicted amino acid sequence of the light and heavy chains of the rodent YFC51.1.1 antibody are shown in SEQ ID NOS: 1 and 2 and SEQ ID NOS: 9 and 10.

Step 2: Designing the humanised antibody

There are several factors to consider in deciding which human antibody sequence to use during the humanisation. The humanisation of light and heavy chains are considered independently of one another, but the reasoning is basically similar for each.

This selection process is based on the following rationale: A given antibody's antigen specificity and affinity is primarily determined by the amino acid sequence of the variable region CDRs. Variable domain framework residues have little or no direct contribution. The primary function of the framework regions is to hold the CDRs in their proper spatial orientation to recognize antigen. Thus the substitution of rodent CDRs into a human

variable domain framework is most likely to result in retention of their correct spatial orientation if the human variable domain framework is highly homologous to the rodent variable domain from which they originated. A human variable domain should preferably be chosen therefore that is highly homologous to the rodent variable domain(s). A suitable human antibody variable domain sequence can be selected as follows:

1. Using a computer program, search all available protein (and DNA) databases for those human antibody variable domain sequences that are most homologous to the rodent antibody variable domains. The output of a suitable program is a list of sequences most homologous to the rodent antibody, the percent homology to each sequence, and an alignment of each sequence to the rodent sequence. This is done independently for both the heavy and light chain variable domain sequences. The above analyses are more easily accomplished if only human immunoglobulin sequences are included.
2. List the human antibody variable domain sequences and compare for homology. Primarily the comparison is performed on length of CDRs, except CDR3 of the heavy chain which is quite variable. Human heavy chains and Kappa and Lambda light chains are divided into subgroups; Heavy chain 3 subgroups, Kappa chain 4 subgroups, Lambda chain 6 subgroups. The CDR sizes within each subgroup are similar but vary between subgroups. It is usually possible to match a rodent antibody CDR to one of the human subgroups as a first approximation of homology. Antibodies bearing CDRs of similar length are then compared for amino acid sequence homology, especially within the CDRs, but also in the surrounding framework regions. The human variable domain which is most homologous is chosen as the framework for humanisation.

Step 3: The actual humanising methodologies/techniques

An antibody may be humanised by grafting the desired CDRs onto a human framework according to EP-A-0239400. A DNA sequence encoding the desired reshaped antibody can therefore be made beginning with the human DNA whose CDRs it is wished to reshape. The rodent variable domain amino acid sequence containing the desired CDRs is compared to that of the chosen human antibody variable domain sequence. The residues in the human variable domain are marked that need to be changed to the corresponding residue in the rodent to make the human variable region incorporate the rodent CDRs. There may also be residues that need substituting in, adding to or deleting from the human sequence.

Oligonucleotides are synthesized that can be used to mutagenize the human variable domain framework to contain the desired residues. Those oligonucleotides can be of any convenient size. One is normally only limited in length by the capabilities of the particular synthesizer one has available. The method of oligonucleotide-directed in vitro mutagenesis is well known.

Alternatively, humanisation may be achieved using the recombinant polymerase chain reaction (PCR) methodology of WO 92/07075. Using this methodology, a CDR may be spliced between the framework regions of a human antibody.

In general, the technique of WO 92/07075 can be performed using a template comprising two human framework regions, AB and CD, and between them, the CDR which is to be replaced by a donor CDR. Primers A and B are used to amplify the framework region AB, and primers C and D used to amplify the framework region CD. However, the primers B and C each also contain, at their 5′ ends, an additional sequence corresponding to all or at least part of the donor CDR sequence. Primers B and C overlap by a length sufficient to permit annealing of their 5′ ends to each other under conditions which allow a PCR to be performed.

Thus, the amplified regions AB and CD may undergo gene splicing by overlap extension to produce the humanised product in a single reaction.

Step 4: The transfection and expression of the reshaped antibody

Following the mutagenesis reactions to reshape the antibody, the mutagenised DNAs can be linked to an appropriate DNA encoding a light or heavy chain constant region, cloned into an expression vector, and transfected into host cells, preferably mammalian cells. These steps can be carried out in routine fashion. A reshaped antibody may therefore be prepared by a process 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 human antibody and the CDRs required for the humanised antibody of the invention;

(b) 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.

Preferably the DNA sequence in step (a) encodes both the variable domain and the or each constant domain of the human antibody chain. The humanised antibody can be prepared using any suitable recombinant expression system. The cell line which is transformed to produce the altered antibody may be a Chinese Hamster Ovary (CHO) cell line or 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.

The CHO cells used for expression of the antibodies according to the invention may be dihydrofolate reductase (dhfr) deficient and so dependent on thymidine and hypoxanthine for growth (Urlaub et al., Proc. Natl. Acac. Sci. U.S.A., 77 4216-4220 (1980)). The parental dhfr CHO cell line is transfected with the DNA encloding the antibody and dhfr which enables selection of CHO cell transformants of dhfr positive phenotype. Selection is carried out by culturing the colonies on media devoid of thymidine and hypoxanthine, the absence of which prevents untransformed cells from growing and transformed cells from resalvaging the folate pathway and thus bypassing the selection system. These transformants usually express low levels of the DNA of interest by virtue of co-integration of transfected DNA of interest and DNA encoding dhfr. The expression levels of the DNA encoding the antibody may be increased by amplification using methotrexate (MTX). This drug is a direct inhibitor of the enzyme dhfr and allows isolation of resistant colonies which amplify their dhfr gene copy number sufficiently to survive under these conditions. Since the DNA sequences encoding dhfr and the antibody are closely linked in the original transformants, there is usually concomitant amplification, and therefore increased expression of the desired antibody.

Another preferred expression system for use with CHO or myeloma cells is the glutamine synthetase (GS) amplification system described in WO 87/04462. This system involves the transfection of a cell with DNA encoding the enzyme GS and with DNA encoding the desired antibody. Cells are then selected which grow in glutamine free medium and can thus be assumed to have integrated the DNA encoding

GS. These selected clones are then subjected to inhibition of the enzyme GS using methionine sulphoximine (Msx). The cells, in order to survive, will amplify the DNA encoding GS with concomitant amplification of the DNA encoding the antibody.

Although the cell line used to produce the humanised 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. The antibody obtained is checked for functionality. If functionality is lost, it is necessary to return to step (2) and alter the framework of the antibody.

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be recovered and 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, a humanised antibody may then be used therapeutically 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 humanised CD18 antibodies can be used for example in the treatment of leukocyte mediated conditions. The humanised CD18 antibodies typically find use in inhibiting influx of leukocytes into the lungs and other organs during sepsis or other infectious or non-infectious trauma. The

humanised CD18 antibody can therefore be used for inhibiting the ingress of leukocytes into the lung and other organs in patients having endotoxic shock or adult respiratory distress syndrome. The antibody can be used to treat asthma or leukocyte-mediated reperfusion damage post thrombolytic therapy, to treat inflammation in the lung and other organs in patients having an inflammation caused by sepsis or other infectious or non-infectious trauma, to eliminate or reduce inflammation in a patient being administered with an anti-infective agent or to assist in the administration of a therapeutic drug to a patient during chemotherapy (EP-A-0346078).

The humanised 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).

The antibodies can also be used as separatelyadministered 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.

An antibody of the present invention may form part of an immunotoxin. 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).

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 cisplatin; 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, "Chimeric Toxins," Olsnes and Phil, Pharmac. Ther., 25, 335-381 (1982), and "Monoclonal Antibodies for Cancer Detection and Therapy," eds. Baldwin and Byers, pp. 159-179, 224-266, Academic Press (1985).

The delivery component of the immunotoxin is a humanised antibody according to 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 IgA, IgM or IgG isotype, but other mammalian constant regions may be utilized as desired.

The invention further provides a pharmaceutical composition comprising a pharmaceutically acceptable

carrier or diluent and, as active ingredient, a humanised antibody according to the invention. The composition may comprise an immunotoxin according to the invention. The humanised antibody, immunotoxin 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 adjustment 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, for example 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).

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. Any suitable 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 or alleviate 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 the 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 CD18 antigen-bearing cells or fragments of the receptor, for vaccine preparation, or the like.

For diagnostic purposes, the antibodies may either be labelled or unlabelled. Unlabelled antibodies can be used in combination with other labelled antibodies (second antibodies) that are reactive with the humanised antibody, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the antibodies can be directly labelled. A wide variety of labels may be employed, such as radionuclides, fluors, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands (particularly haptens), etc. Numerous types of immunoassays are available and are well known to those skilled in the art.

Kits can also be supplied for use with the subject antibodies in the protection against or detection of a cellular activity or for the presence of a selected antigen. Thus, a humanised antibody 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. 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 kit will generally also include a set of instructions for use.

The following Example illustrates the invention.

EXAMPLE

Cloning and sequencing of the YFC51.1.1 rat anti-human-CD18 heavy and light chains

Total RNA was isolated from 2.5 x 10^7 YFC51.1.1 expressing cells following the method of Chomczynski and Sacchi (Anal. Biochem., 162, 156-159, (1987)), using 1ml of extraction solution per 1 x 10^7 cells. The resulting RNA pellet was redissolved in 50μl diethyl pyrocarbonate (DEPC)-treated distilled water, and spectrophotometrically determined to be at a concentration of 4μg/μl. Dynabeads Oligo $(dT)_{25}$ (Dynal) was used to extract mRNA from 75μg total RNA employing the manufacturer's protocol.

cDNA was synthesized from the isolated mRNA and cloned into the plasmid pSPORT-1 using the SUPERSCRIPT Plasmid System for cDNA Synthesis and Plasmid Cloning kit (BRL) following the method recommended by the manufacturer. Escherichia coli MAX EFFICIENCY DH5α Competent Cells (BRL) were transformed with the resulting cDNA/pSPORT-1 ligation. Approximately 5000 colonies were lifted onto Hybond-N nylon filters (Amersham) and lysed, denatured and fixed following the method of Buluwela et al (Nucleic Acids Res., 17, 452, (1989)). The filters were treated with proteinase K (50μg//ml) in 0.2 x SSC, 0.1% SDS at 55°C for 30 min, and then excess debris removed with a tissue.

(i) Heavy chain

An oligonucleotide as shown in SEQ ID NO: 17 complementary to a portion of rat gamma-CH1 constant region (bases 496-515) was end-labelled and used to screen the filters for YFC51.1.1 heavy chain following the standard protocols. Approximately 50 potential positive colonies were detected, and 20 selected for further analysis. Plasmid DNA was prepared using the method of Del Sal et al (Nucleic Acids Res., 16, 9878, (1988)) and 12 of the 20 contained inserts of the expected size for rat immunoglobulin heavy chain cDNA. A clone, p51H.6, was selected, and the variable region sequenced in both directions by plasmid priming following the dideoxy chain termination method (Sanger et al, (Proc. Natl. Acad. Sci., USA, 74, 5463-5467, (1977)), according to the Sequenase kit (USB) protocol. The sequence of the variable region is shown in SEQ ID NOS: 9 and 10.

(ii) Light Chain

A clone of the rat myeloma Y3-Ag 1.2.3 light chain (Crowe et al, Nucleic Acid Res., 17, 7992, (1989)) was labelled with digoxigenin-11-dUTP using the Nonradioactive DNA labelling and Detection Kit (Boehringer Mannheim) and used to screen the filters for the YFC51.1.1 light chain,

following the manufacturer's protocol. Approximately 40 potential positive colonies were detected, and 24 selected for further analysis. Plasmid DNA was prepared as described above. Both Y3-Ag 1.2.3 and YFC51.1.1 light chains were isolated (Y3 cell line being hybridoma fusion partner) but were distinguishable by having different restriction patterns. One clone, p51L.4, containing the YFC51.1.1 light chain was chosen and sequenced as described for the heavy chain. The sequence of the variable region is shown in SEQ ID NOS: 1 and 2.

Designing the humanised antibody

Using the selection procedure described in Step (2) above, the human variable domain frameworks of the NEWM heavy chain and REI light chain (Kabat et al, 1987) were chosen for the humanisation process.

Construction of the humanised heavy and light chain genes

The humanised heavy and light chains were constructed following the method of Lewis and Crowe (Gene, 101, 297-302, (1991)).

(i) Light Chain

Light chain oligonucleotide primers:

A_L: SEQ ID NO: 18:
B_L: SEQ ID NO: 19:
C_L: SEQ ID NO: 20:
D_L: SEQ ID NO: 21:
E_L: SEQ ID NO: 22:
F_L: SEQ ID NO: 23:
G_L: SEQ ID NO: 24:
H_L: SEQ ID NO: 25:

PCR reactions (Saiki et al., Science 239, 487-491, (1988)) were performed in a programmable heating block (Hybaid) using 20 rounds of temperature cycling (94°C for 1 min, 50°C for 2 min, and 72°C for 3 min) followed by a

final 10 min step at 72°C. 1μg of each primer, a specified amount of template, and 2.5 units of Taq polymerase (Perkin Elmer Cetus) were used in a final volume of 100 μl with the reaction buffer as recommended by the manufacturer.

The initial template for the PCR was CAMPATH-1H light chain (humanised CAMPATH-1 on RE1 framework; Page and Sydenham, Biotechnology, 9, 64-68, (1991)). Four initial PCR reactions were carried out, with 10ng of template per reaction, using the primer pairs A_L with B_L, C_L with D_L, E_L with F_L, and G_L with H_L respectively. The products of these PCR reactions, fragments AB_L, CD_L, EF_L and GH_L respectively, were purified using Prep-A-Gene (Bio-Rad) following the protocol recommended by the manufacturer. Fragments AB_L with CD_L, and EF_L with GH_L were combined using a quarter of each purified product, and subjected to recombinant PCR reactions with primers A_L plus D_L, and E_L plus H_L respectively. The products of these reactions, fragments AD_L and EH_L, were purified as above, and a quarter of each combined in a recombinant PCR reaction using primers A_L and H_L. The final humanised light chain recombinant PCR product, AH_L, was cloned into the HindIII site of pUC-18 (BRL) following the method of Crowe et al., Nucleic Acids Res., 19, 184, (1991), utilising the HindIII sites in primers A_L and H_L. Plasmid isolates were sequenced by the dideoxy chain termination method, and clones of the correct sequence chosen.

(ii) Heavy Chain

Heavy chain oligonucleotide primers:

A_H: . SEQ ID NO: 26:

B_H: . SEQ ID NO: 27:

C_H: . SEQ ID NO: 28:

D_H: . SEQ ID NO: 29:

E_H: . SEQ ID NO: 30:

F_H: . SEQ ID NO: 31:

G_H: . SEQ ID NO: 32:

H_H: . SEQ ID NO: 33:

The initial template for the PCR was CAMPATH-1H heavy chain. The rodent CDR's were grafted on to the template following the recombinant PCR method as described above, but using oligonucleotide primers A_H to H_H. The final PCR, i.e. fragments AD_H and EH_H with primers A_H and H_H, did not give a high yield of product so a fragment AF_H was generated (from fragments AD_H and EF_H) and used with fragment EH_H in a PCR with primers A_H and H_H. Oligonucleotides A_H and H_H were designed with HindIII and EcoRI sites respectively to enable initial cloning of the humanised variable region, and a SpeI site was introduced into the NEWM framework 4 (FR4) region of oligonucleotide G_H to facilitate subsequent cloning of the variable region with a suitable constant region of choice. The SpeI site was chosen so as not to alter the leucine residue at position 109 (numbering according to Kabat et al, 1987) of the humanised heavy chain template. Four out of the six human heavy J-minigenes possess a leucine at this position (Kabat et al, 1987). Thus the use of the engineered SPeI site should be generally applicable.

The humanised heavy chain variable region recombinant PCR product was cloned into HindIII/EcoRI-cut pUC-18 (BRL), and plasmid isolates of the correct sequence were chosen. The FR4 and γ1 constant regions of CAMPATH-1H heavy chain were PCR cloned into pUC-18 (BRL) using oligonucleotide primers X_H (SEQ ID NO: 34) and Y_H (SEQ ID NO: 35). Primer X_H contains SpeI and HindIII sites, and Y_H an EcoRI site. The HindIII and ECoRI sites were used to clone the PCR product into pUC-18, and plasmid isolates of the correct sequence were selected. The complete heavy chain was subsequently reconstituted from the humanised variable region and γ1 constant region clones using the engineered FR4 Spel site.

Transient expression in COS cells

DNA encoding the humanised heavy and light chains were cloned into the vectors pEE6.hCMV and pEE12 respectively, see Stephens & Cockett, Nucleic Acids Res., 17, 7110 (1989); Bebbington et al., Biotechnology, 10, 169 (1992); and Bebbington and Hentschel in Glover ed., DNA Cloning Volume III, Academic Press (1987). The vector pEE12 is a pBR322 - based vector containing the h-CMV-MEI promoter and the hamster glutamine synthetase (GS) cDNA under control of the SV40 early region promoter. The vector pEE12 corresponds to pEE6 (see EP-A-0338841) with the GS cDNA expression cassette driven by the SV40 promoter transcribing in the same direction as the h-CMV-MEI promoter. Cells transfected with the vectors pEE6, hCMV and pEE12 are capable of growth in glutamine free medium because of the presence of the GS cDNA. As the selection is only on the pEE12 plasmid, effective expression relies upon co-integration of both plasmids.

The recombinant plasmids (5μg of each) were transfected into $5x10^5$ COS-1 cells using the Transfectam reagent (Promega, Southampton, U.K.) under the conditions recommended by the manufacturer. Stock COS-1 cells (source ECACC, Porton Down, U.K.) were maintained in DMEM medium (Flow, Irvine, U.K.) supplemental with 10% foetal calf serum (APP, Dudley, U.K.). COS cell transfections were carried out in DMEM medium (Flow, Irvine, U.K.). Growth media from COS-1 cells four days post transfection were assayed by a sandwich ELISA assay using flexible microtitre plates (Falcon, Becton-Dickinson, Plymouth, U.K.) coated with polyclonal anti-human IgG (Sigma, Poole, U.K.) as capture antibody. The assay sample was added and detection performed with an anti-human IgG γ chain-specific peroxidase conjugate (Seralab, Crawley Down, U.K.) and orthophenylene dimine-HCl (Sigma, Poole U.K.) as substrate.

The humanised antibody was shown to be expressed transiently in the COS cells by using the spent COS cell supernatant to surface label MF-14 (a T-cell clone) cells

for FACS analysis according to the method of Gladwin et al, Biochem. Biophs. Acta, 1052, 166-172 (1990). Briefly 100µl aliquots of a cell suspension (10^5) were incubated with the appropriate antibody (spent COS cell supernatant) and incubated on melting ice for 30 minutes. The cells were washed twice in PBS and incubated for a further 30 minutes with the appropriate second antibody (see below). The cells were washed again and 1:50 dilutions of anti-rat Ig-FITC or anti-human Ig-FITC conjugates were added on melting ice. Finally, the cells were washed three times in PBS and fixed in 0.1% paraformaldehyde. Analysis of surface labelling was performed using a Becton-Dickenson FACScan using standard computer, electronics and optics.

The humanised antibody in the COS cell supernatant was shown to bind MF-14 cells as well as inhibiting the binding of the rat YFC51.1.1 monoclonal antibody. Since the humanised antibody was shown to have retained binding for CD18 by blocking the binding of the rat monoclonal antibody, stable NSO transfactants were generated.

Stable expression in NSO cells

A single expression vector for generating stable transfectants of NSO cells was generated by cloning the complete heavy chain expression cassette from pEE6 into the BamHI site of the pEE12 - light chain plasmid. Thus both heavy and light chain coding sequences are transcribed in the same direction from the same vector. 40µg of plasmid for transfection was linearised by digestion with SalI restriction enzyme that has a recognition sequence within the bacterial plasmid sequence. The linearised DNA was precipitated from solution using ethanol, washed in 70% ethanol, dried and resuspended in sterile water.

Exponentially growing NSO cells (a Human mycloma cell line; see Jarvis, Methods in Enzymology, 73B, 3 (1981); source ECACC , Porton Down, U.K.) were maintained in non-selective DMEM medium (i.e. without glutamine and ferric

nitrate but with sodium pyruvate at 110 mg/l (GIBCO/BRL, Paisley, U.K.) supplemented with 1X non-essential amino acids (Flow, Irvine, U.K.) 2mM glutamine (GIBCO) and 10% foetal calf serum (APP, Dudley, U.K.). NSO cells were centrifuged, washed and re-suspended in cold PBS, such that after the addition of the DNA the cells would be at a concentration of 10^7 cells/ml. The linearised plasmid DNA, 40μg, was added to 10^7 cells in an electroporation cuvette on ice. The cells and DNA were mixed gently so as to avoid generating bubbles and the mixture was left on ice for 5 minutes. The outside of the cuvette was wiped dry and two consecutive pulses at 1500V, 3mF were delivered using a Gene Pulser (Bio-Rad). The cuvette was returned to ice for 5 minutes.

Transfected cells were transferred to 96 well plates at densities of 3×10^5, 7.5×10^4 and 1.5×10^4 cells/ml in 50μl of non-selective medium and incubated at 37°C for 24 hours. Subsequently 100 μl of selective DMEM medium (i.e. without glutamine and ferric nitrate but with sodium pyruvate at 100 mg/l (GIBCO/BRL, Paisley, U.K.) supplemented with glutamate (60 mg/ml), asparagine (60 mg/ml; Sigma, Poole, U.K.), 1X non-essential amino acids, 7 mg/l of adenosine, cytidine, guanosine and uridine, 2.4 mg/l of thymidine (Sigma, Poole, U.K.) and 10% dialysed foetal calf serum (APP, Dudley U.K.)) was added to selected clones which had integrated the transfected plasmid. The plates were returned to the incubator and left until substantial cell death had occurred and discrete surviving colonies had appeared. Once colonies of glutamine-independent transfectants could be seen, wells with single colonies were selected and spent tissue culture supernatants were collected and assayed for human IgG secretion.

Wells with single colonies that were positive for IgG secretion were then expanded in culture using selective medium. The cells were distributed in 96 well plates at 10^4

cells/well in 100µl of medium and incubated overnight. 100µl of selective medium containing a concentration of L-methionine sulphoximine (MSX) was added. MSX is a toxic glutamine analogue that allows for selection of vector amplification. Each 96-well plate had a different final concentration of MSX, ranging from 200µM down to 12.5µM. Individual colonies were isolated from each independent transfectant at the highest MSX concentration at which MSX resistance occurred. The colonies were expanded and antibody secretion rate (in $\mu g/10^6$ cells/day) was compared with the unamplified rate. Clones were obtained that expressed the humanised antibody at 1 to 3 $\mu g/10^6$ cells/day.

The humanised antibody was purified from spent tissue culture supernatant by affinity chromatography over a Superose protein-G column (Pharmacia) and used in T-cell proliferation assays and C1q binding studies.

T-cell Proliferation

Peripheral human mono-nuclear cells were isolated from defibrinated whole human blood using Lymphoprep (Nycomed, Oslo, Norway) and following the manufacturer's protocol. Triplicate cultures were set up in 96 well flat bottomed microtitre plates (Nunclon, Roskild, Denmark) with the medium clone (RPMI 1640 supplemented with 10% autologous serum, 2mM glutamine and 100 IU/ml penicillin, 100µg/ml streptomycin) or with medium and antigen (Tetanus toxoid, 5µg/ml) or medium and mitogen (PHA, 5µg/ml), in the presence or absence ofYFC 51.1.1 or the humanised antibody. Cells were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO_2 for five days. Wells were then pulsed with 1µCi [methyl ^{3}H]thymidine (2Ci/mmol, Amersham), harvested 4 hours later and radioactivity counted by liquid scintillation using a ß counter (LKB, Betaplate, Sweden).

Both the rat YFC51.1.1 monoclonal antibody and the humanised antibody strongly inhibited the antigen specific

T-cell response but had little effect on the PHA induced proliferation. However, at high levels of antibody (50μg/ml) and low levels of PHA (2.5μg/ml) up to 80% inhibition could be obtained.

Complement binding

Human mononuclear cells (prepared as above) were stimulated with PHA at 5μg/ml and incubated at 37°C for 3 days. The PHA was removed by washing the cells in PBS. The cells were then incubated with 10μg/ml of test antibody for 20 minutes on ice, cells washed in ice cold PBS and incubated with ice cold human serum for 20 minutes. The human serum was removed by washing in ice cold PBS. The cells were then incubated for 20 minutes with a fluoreceinated polyclonal sheep anti-human C1q. Unbound anti-C1q was removed by washing cells in PBS and cells were analysed on a Becton-Dickenson FACScan. YFC51.1.1 was found to bind human C1q weakly and no binding was detected for the humanised antibody. Potential therapeutic uses for anti-CD18 antibodies rely on transient inhibition of CD18-mediated adherence of leukocytes rather than depletion of CD18 positive cells. Accordingly the inability of the humanised antibody to fix human complement on CD18 positive cells is an advantage since it suggests that in vivo the antibody will not deplete using complement but will function as a blocking antibody.

FACS analysis

A CD18 positive T-cell clone (MF14) was used to determine the binding of humanised compared with rat antibody. Cells were incubated with rat or humanised antibody for 30 minutes on ice. Unbound antibody was removed by washing and the second antibody was added (i.e. rat antibody was added to cells pre-incubated with humanised antibody and vice versa) and incubated for 30 minutes on ice. Cells were washed to remove unbound

antibody and a FITC-labelled anti-human or anti-rat antibody added. Unbound label was removed by washing and the cells were analysed on a Becton-Dickenson FACScan. Pre-incubation of MF14 cells with 10μg/ml of YFC51.1.1 antibody completely blocked the binding of 0.1μg/ml of humanised antibody. In the reciprocal experiment, pre-incubation with 10μg/ml of humanised antibody completely blocked the binding of 0.1μg/ml YFC51.1.1. In both cases use of 1.0 and 0.1μg/ml of the first antibody led to a titration of blocking.

SEQUENCE LISTING

(1) INFORMATION FOR SEQ ID NO : 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 375 base pairs
(B) TYPE : nucleic acid
(C) STRANDEDNESS : double
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM : Rattus rattus

(ix) FEATURE:

(A) NAME/KEY : CDS
(B) LOCATION : 1..375
(D) OTHER INFORMATION : /product= "Variable region light chain"
/standard_name= "YFC51.1.1"

(ix) FEATURE:

(A) NAME/KEY : misc_signal
(B) LOCATION : 1..60
(D) OTHER INFORMATION : /function= "Signal Sequence"

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 130..162

(D) OTHER INFORMATION : /function= "CDR 1"

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 208..228
(D) OTHER INFORMATION : /function= "CDR 2"

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 325..351
(D) OTHER INFORMATION : /function= "CDR 3"

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

```
ATG AGG GTC CAG GTT CAG TTT CTG GGG CTC CTT CTG CTC TGG ACA TCA    48
Met Arg Val Gln Val Gln Phe Leu Gly Leu Leu Leu Trp Thr Ser
 1               5                  10                  15

GGT GCC CAG TGT GAT GTC CAG ATG ACC CAG TCT CCG TCT TAT CTT GCT    96
Gly Ala Gln Cys Asp Val Gln Met Thr Gln Ser Pro Ser Tyr Leu Ala
            20                  25                  30

GCG TCT CCT GGA GAA AGT GTT TCC ATC AGT TGC AAG GCA AGT AAG AGC   144
Ala Ser Pro Gly Glu Ser Val Ser Ile Ser Cys Lys Ala Ser Lys Ser
        35                  40                  45

ATT AGC AAT TAT TTA GCC TGG TAT CAA CAG AAA CCT GGG GAA GCA AAT   192
Ile Ser Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Glu Ala Asn
    50                  55                  60

AAA CTT CTT GTC TAT TAT GGG TCA ACT TTG CGA TCT GGA ATT CCA TCG   240
Lys Leu Leu Val Tyr Tyr Gly Ser Thr Leu Arg Ser Gly Ile Pro Ser
65                  70                  75                  80

AGG TTC AGT GGC AGT GGA TCT GGT ACA GAT TTC ACT CTC ACC ATC AGA   288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Arg
                85                  90                  95
```

```
AAC CTG GAG CCT GCA GAT TTT GCA GTC TAC TAC TGT CAA CAG TAT TAT   336
Asn Leu Glu Pro Ala Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr
            100                 105                 110

GAA AGA CCG CTC ACG TTC GGT TCT GGG ACC AAG CTG GAG               375
Glu Arg Pro Leu Thr Phe Gly Ser Gly Thr Lys Leu Glu
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO : 2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 125 amino acids
(B) TYPE : amino acid
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : protein

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

```
Met Arg Val Gln Val Gln Phe Leu Gly Leu Leu Leu Leu Trp Thr Ser
 1               5                  10                  15

Gly Ala Gln Cys Asp Val Gln Met Thr Gln Ser Pro Ser Tyr Leu Ala
            20                  25                  30

Ala Ser Pro Gly Glu Ser Val Ser Ile Ser Cys Lys Ala Ser Lys Ser
        35                  40                  45

Ile Ser Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Glu Ala Asn
    50                  55                  60

Lys Leu Leu Val Tyr Tyr Gly Ser Thr Leu Arg Ser Gly Ile Pro Ser
65                  70                  75                  80

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

Asn Leu Glu Pro Ala Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr
            100                 105                 110
```

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

(3) INFORMATION FOR SEQ ID NO : 3

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 33 base pairs
(B) TYPE : nucleic acid
(C) STRANDEDNESS : double
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM : Rattus rattus

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 1..33
(D) OTHER INFORMATION : /function= "CDR 1"

(ix) FEATURE:

(A) NAME/KEY : CDS
(B) LOCATION : 1..33

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

AAG GCA AGT AAG AGC ATT AGC AAT TAT TTA GCC 33
Lys Ala Ser Lys Ser Ile Ser Asn Tyr Leu Ala
1 5 10

(4) INFORMATION FOR SEQ ID NO : 4

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 11 amino acids
(B) TYPE : amino acid
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : protein

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

Lys Ala Ser Lys Ser Ile Ser Asn Tyr Leu Ala
1 5 10

(5) INFORMATION FOR SEQ ID NO : 5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 21 base pairs
(B) TYPE : nucleic acid
(C) STRANDEDNESS : double
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM : Rattus rattus

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 1..21
(D) OTHER INFORMATION : /function= "CDR 2"

(ix) FEATURE:

(A) NAME/KEY : CDS
(B) LOCATION : 1..21

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

TAT GGG TCA ACT TTG CGA TCT 21
Tyr Gly Ser Thr Leu Arg Ser
1 5

(6) INFORMATION FOR SEQ ID NO : 6

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 7 amino acids
(B) TYPE : amino acid
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : protein

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

Tyr Gly Ser Thr Leu Arg Ser
1 5

(7) INFORMATION FOR SEQ ID NO : 7

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 27 base pairs
(B) TYPE : nucleic acid
(C) STRANDEDNESS : double
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM : Rattus rattus

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 1..27
(D) OTHER INFORMATION : /function= "CDR 3"

(ix) FEATURE

(A) NAME/KEY : CDS

(B) LOCATION : 1..27

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

```
CAA CAG TAT TAT GAA AGA CCG CTC ACG                                    27
Gln Gln Tyr Tyr Glu Arg Pro Leu Thr
 1               5
```

(8) INFORMATION FOR SEQ ID NO : 8

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 9 amino acids
(B) TYPE : amino acid
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : protein

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

```
Gln Gln Tyr Tyr Glu Arg Pro Leu Thr
 1               5
```

(9) INFORMATION FOR SEQ ID NO : 9

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 417 base pairs
(B) TYPE : nucleic acid
(C) STRANDEDNESS : double
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM : Rattus rattus

(ix) FEATURE:

(A) NAME/KEY : CDS
(B) LOCATION : 1..417
(D) OTHER INFORMATION : /product= "Heavy chain variable region with signal sequence"
/standard_name "YFC51.1.1"

(ix) FEATURE:

(A) NAME/KEY : misc_signal
(B) LOCATION : 1..57
(D) OTHER INFORMATION : /function= "Signal sequence"

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 148..162
(D) OTHER INFORMATION : /function= "CDR 1"

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 205..255

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 352..384

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

```
ATG AAA TGC AGC TGG ATC AAC CTC TTC TTG ATG GCA CTA GCT TCA GGG     48
Met Lys Cys Ser Trp Ile Asn Leu Phe Leu Met Ala Leu Ala Ser Gly
 1               5                  10                  15

GTC TAC GCA GAA GTG CAG CTG CAA CAG TCT GGG CCC GAG CTT CGG AGA     96
Val Tyr Ala Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Arg Arg
            20                  25                  30

CCT GGG TCC TCA GTC AAG TTG TCT TGT AAG ACT TCT GGC TAC AGC ATT    144
Pro Gly Ser Ser Val Lys Leu Ser Cys Lys Thr Ser Gly Tyr Ser Ile
        35                  40                  45

AAA GAT TAC CTT CTG CAC TGG GTA AAA CAT AGG CCA GAA TAC GGC CTG    192
Lys Asp Tyr Leu Leu His Trp Val Lys His Arg Pro Glu Tyr Gly Leu
    50                  55                  60

GAA TGG ATA GGA TGG ATT GAT CCT GAG GAT GGT GAA ACA AAG TAT GGT    240
Glu Trp Ile Gly Trp Ile Asp Pro Glu Asp Gly Glu Thr Lys Tyr Gly
65                  70                  75                  80

CAG AAG TTT CAA AGC AGG GCC ACA CTC ACT GCA GAT ACA TCC TCC AAC    288
Gln Lys Phe Gln Ser Arg Ala Thr Leu Thr Ala Asp Thr Ser Ser Asn
                85                  90                  95

ACA GCC TAC ATG CAA CTC AGC AGC CTG ACG TCT GAC GAC ACA GCA ACC    336
Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Asp Asp Thr Ala Thr
            100                 105                 110

TAT TTT TGT ACT AGA GGC GAA TAT AGA TAC AAC TCG TGG TTT GAT TAC    384
```

```
Tyr Phe Cys Thr Arg Gly Glu Tyr Arg Tyr Asn Ser Trp Phe Asp Tyr
        115                 120                 125

TGG GGC CAA GGC ACT CTG GTC ACT GTC TCT TCA                      417
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
    130                 135
```

(10) INFORMATION FOR SEQ ID NO : 10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 139 amino acids
(B) TYPE : amino acid
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : protein

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

```
Met Lys Cys Ser Trp Ile Asn Leu Phe Leu Met Ala Leu Ala Ser Gly
 1               5                  10                  15

Val Tyr Ala Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Arg Arg
            20                  25                  30

Pro Gly Ser Ser Val Lys Leu Ser Cys Lys Thr Ser Gly Tyr Ser Ile
        35                  40                  45

Lys Asp Tyr Leu Leu His Trp Val Lys His Arg Pro Glu Tyr Gly Leu
    50                  55                  60

Glu Trp Ile Gly Trp Ile Asp Pro Glu Asp Gly Glu Thr Lys Tyr Gly
65                  70                  75                  80

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

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

Tyr Phe Cys Thr Arg Gly Glu Tyr Arg Tyr Asn Ser Trp Phe Asp Tyr
```

40

115 120 125

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
130 135

(11) INFORMATION FOR SEQ ID NO : 11

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 15 base pairs
(B) TYPE : nucleic acid
(C) STRANDEDNESS : double
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM : Rattus rattus

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 1..15
(D) OTHER INFORMATION : /function= "CDR 1"

(ix) FEATURE:

(A) NAME/KEY : CDS
(B) LOCATION : 1..15

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

GAT TAC CTT CTG CAC 15
Asp Tyr Leu Leu His
1 5

(12) INFORMATION FOR SEQ ID NO : 12

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 5 amino acids
(B) TYPE : amino acid
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : protein

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

Asp Tyr Leu Leu His
1 5

(13) INFORMATION FOR SEQ ID NO : 13

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 51 base pairs
(B) TYPE : nucleic acid
(C) STRANDEDNESS : double
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM : Rattus rattus

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 1..51
(D) OTHER INFORMATION : /function= "CDR 2"

(ix) FEATURE:

(A) NAME/KEY : CDS
(B) LOCATION : 1..51

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

```
TGG ATT GAT CCT GAG GAT GGT GAA ACA AAG TAT GGT CAG AAG TTT CAA    48
Trp Ile Asp Pro Glu Asp Gly Glu Thr Lys Tyr Gly Gln Lys Phe Gln
 1               5                  10                  15

AGC                                                               51
Ser
```

(14) INFORMATION FOR SEQ ID NO : 14

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 17 amino acids
(B) TYPE : amino acid
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : protein

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

```
Trp Ile Asp Pro Glu Asp Gly Glu Thr Lys Tyr Gly Gln Lys Phe Gln
 1               5                  10                  15

Ser
```

(15) INFORMATION FOR SEQ ID NO : 15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 33 base pairs
(B) TYPE : nucleic acid
(C) STRANDEDNESS : double
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM : Rattus rattus

(ix) FEATURE:

(A) NAME/KEY : misc_feature
(B) LOCATION : 1..33
(D) OTHER INFORMATION : /function= "CDR 3"

(ix) FEATURE:

(A) NAME/KEY : CDS
(B) LOCATION : 1..33

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

```
GGC GAA TAT AGA TAC AAC TCG TGG TTT GAT TAC                    33
Gly Glu Tyr Arg Tyr Asn Ser Trp Phe Asp Tyr
 1               5                  10
```

(16) INFORMATION FOR SEQ ID NO : 16

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 11 amino acids
(B) TYPE : amino acid
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : protein

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

Gly Glu Tyr Arg Tyr Asn Ser Trp Phe Asp Tyr
1 5 10

(17) INFORMATION FOR SEQ ID NO : 17

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 20 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : cDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : NO

(vi) ORIGINAL SOURCE

(A) ORGANISM : Rattus Rattus

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

AGTGGATAGA CAGATGGGGC 20

(18) INFORMATION FOR SEQ ID NO : 18

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 30 bases

(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : NO

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

GATCAAGCTT CTCTACAGTT ACTGAGCACA 30

(19) INFORMATION FOR SEQ ID NO : 19

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 43 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : YES

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

GCTAAATAAT TGCTAATGCT CTTACTTGCT TTACAGGTGA TGG 43

(20) INFORMATION FOR SEQ ID NO : 20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 43 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : NO

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

AGAGCATTAG CAATTATTTA GCCTGGTACC AGCAGAAGCC AGG 43

(21) INFORMATION FOR SEQ ID NO : 21

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 41 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : YES

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

AGATCGCAAA GTTGACCCAT AGTAGATCAG CAGCTTTGGA G 41

(22) INFORMATION FOR SEQ ID NO : 22

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 41 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : NO

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

TATGGGTCAA CTTTGCGATC TGGTGTGCCA AGCAGATTCA G 41

(23) INFORMATION FOR SEQ ID NO : 23

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 47 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : YES

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

CGTGAGCGGT CTTTCATAAT ACTGTTGGCA GTAGTAGGTG GCGATGT 47

(24) INFORMATION FOR SEQ ID NO : 24

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH	: 47 bases
(B) TYPE	: nucleic acid
(C) STRANDEDNESS	: single
(D) TOPOLOGY	: linear
(ii) MOLECULE TYPE	: ssDNA
(iii) HYPOTHETICAL	: NO
(iv) ANTI-SENSE	: NO
(xi) SEQUENCE DESCRIPTION	: SEQ ID NO: 24:

CAACAGTATT ATGAAAGACC GCTCACGTTC GGCCAAGGGA CCAAGGT 47

(25) INFORMATION FOR SEQ ID NO : 25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH	: 30 bases
(B) TYPE	: nucleic acid
(C) STRANDEDNESS	: single
(D) TOPOLOGY	: linear
(ii) MOLECULE TYPE	: ssDNA
(iii) HYPOTHETICAL	: NO

(iv) ANTI-SENSE : YES

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

GATCAAGCTT CTAACACTCT CCCCTGTTGA 30

(26) INFORMATION FOR SEQ ID NO : 26

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 31 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : NO

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

TGGGATCGAT CAAGCTTTAC AGTTACTGAG C 31

(27) INFORMATION FOR SEQ ID NO : 27

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 36 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : YES

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

GTGCAGAAGG TAATCGGTGA AGGTGAAGCC AGACAC 36

(28) INFORMATION FOR SEQ ID NO : 28

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 36 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : NO

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

GATTACCTTC TGCACTGGGT GAGACAGCCA CCTGGA 36

(29) INFORMATION FOR SEQ ID NO : 29

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 54 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : YES

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

ATACTTTGTT TCACCATCCT CAGGATCAAT CCATCCAATC CACTCAAGAC CTCG 54

(30) INFORMATION FOR SEQ ID NO : 30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 54 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : NO

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

GGTGAAACAA AGTATGGTCA GAAGTTTCAA AGCAGAGTGA CAATGCTGGT AGAC 54

(31) INFORMATION FOR SEQ ID NO : 31

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 45 bases
(B) TYPE : nucleic acid

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

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : YES

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

CCACGAGTTG TATCTATATT CGCCTCTTGC ACAATAATAG ACCGC 45

(32) INFORMATION FOR SEQ ID NO : 32

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 54 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : NO

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

AGATACAACT CGTGGTTTGA TTACTGGGGT CAAGGCTCAC TAGTCACAGT CTCC 54

(33) INFORMATION FOR SEQ ID NO : 33

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 36 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : YES

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

TAGAGTCCTG AGGGAATTCG GACAGCCGGG AAGGTG 36

(34) INFORMATION FOR SEQ ID NO : 34

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 48 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : NO

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 34:

GCTGCTCCTT TTAAGCTTTG GGGTCAAGGC TCACTAGTCA CAGTCTCC 48

(35) INFORMATION FOR SEQ ID NO : 35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 33 bases
(B) TYPE : nucleic acid
(C) STRANDEDNESS : single
(D) TOPOLOGY : linear

(ii) MOLECULE TYPE : ssDNA

(iii) HYPOTHETICAL : NO

(iv) ANTI-SENSE : YES

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 35:

AAGCTTCCGT CGAATTCATT TACCCGGAGA CAG 33

CLAIMS:

1. A humanised antibody in which sufficient of the amino acid sequence of each CDR shown below is provided such that the antibody is capable of binding to the human CD-18 antigen:

light chain: CDR1 (SEQ ID NOS: 3 and 4)
CDR2 (SEQ ID NOS: 5 and 6)
CDR3 (SEQ ID NOS: 7 and 8)
heavy chain: CDR1 (SEQ ID NOS: 11 and 12)
CDR2 (SEQ ID NOS: 13 and 14)
CDR3 (SEQ ID NOS: 15 and 16).

2. An antibody as claimed in claim 1, in which the variable domain framework of the light chain is or is substantially homologous to the variable domain framework of the protein REI.

3. An antibody as claimed in claim 1 or 2, in which the variable domain framework of the heavy chain is or is substantially homologous to the variable domain framework of the protein NEWM.

4. An antibody as claimed in any one of claims 1 to 3 in which the CDRs are the light chain CDRs 1 to 3 and the heavy chain CDRs 1 to 3 specified in claim 1.

5. A process for the preparation of a humanised antibody as defined in any of claims 1 to 4, which process comprises providing a host transformed with either (i) a first expression vector which encodes the light chain of the humanised antibody and a second expression vector which encodes the heavy chain of the humanised antibody; or (ii) a single expression vector which encodes both the light chain and the heavy chain of the humanised antibody; and maintaining said host under such conditions that each chain is expressed and isolating the humanised antibody formed by assembly of the thus-expressed chains.

6. A DNA molecule encoding a humanised antibody in

which sufficient of the amino acid sequence of each CDR shown below is provided such that the antibody is capable of binding to the human CD-18 antigen:

light chain: CDR1 (SEQ ID NOS: 3 and 4)
CDR2 (SEQ ID NOS: 5 and 6)
CDR3 (SEQ ID NOS: 7 and 8)
heavy chain: CDR1 (SEQ ID NOS: 11 and 12)
CDR2 (SEQ ID NOS: 13 and 14)
CDR3 (SEQ ID NOS: 15 and 16).

7. A DNA molecule as claimed in claim 6, in which the variable domain framework of the light chain is or is substantially homologous to the variable domain framework of the protein REI.

8. A DNA molecule as claimed in claim 6 or 7, in which the variable domain framework of the heavy chain is or is substantially homogenous to the variable domain framework of the protein NEWM.

9. A DNA molecule as claimed in any one of claims 6 to 8 in which the CDRs are the light chain CDRs 1 to 3 and the heavy chain CDRs 1 to 3 specified in claim 6.

10. A DNA molecule as claimed in any of claims 6 to 9 in the form of an expression vector.

11. A host transformed with an expression vector as claimed in claim 10.

12. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, a humanised antibody as defined in any of claims 1 to 4.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01289

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; C07K15/28; C12N5/10
A61K39/395

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C12P ; C07K ; A61K

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

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	NUCLEIC ACIDS RESEARCH vol. 19, no. 9, 11 May 1991, LONDON, GB pages 2471 - 2476 B. DAUGHERTY ET AL. 'Polymerase chain reaction facilitates the cloning, CDR-grafting, and rapid expression of a murine monoclonal antibody directed against the CD18 component of leukocyte integrins.' see the whole document --- -/--	1-12

[10] Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 SEPTEMBER 1992	19. 10. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	NOOIJ F.J.M.

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

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

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	GENE vol. 101, no. 2, 30 May 1991, AMSTERDAM, THE NETHERLANDS pages 297 - 302 A. LEWIS ET AL. 'Immunoglobulin complementarity-determining region grafting by recombinant polymerase chain reaction to generate humanised monoclonal antibodies.' cited in the application see the whole document	1-12
Y	EP,A,0 346 078 (THE ROCKEFELLER UNIVERSITY) 13 December 1989 cited in the application see claims	1-12
Y	(EDS. W. KNAPP ET AL.) 'Leukocyte Typing IV. White cell differentiation antigens' 1989 , OXFORD UNIVERSITY PRESS , OXFORD see page 1079	1-12
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, no. 10, 15 May 1991, WASHINGTON DC, US pages 4181 - 4185 S. GORMAN ET AL. 'Reshaping a therapeutic CD4 antibody.' see abstract	1-12
P,X	EP,A,0 438 312 (MERCK & CO., INC.) 24 July 1991 see claims	1-12

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201289 SA 62146

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/09/92
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
EP-A-0346078	13-12-89	AU-B- 620100	13-02-92
		AU-A- 3608489	14-12-89
		JP-A- 2104534	17-04-90
EP-A-0438312	24-07-91	AU-A- 6984391	25-07-91
		CA-A- 2034574	20-07-91
		EP-A- 0440351	07-08-91

EPO FORM P079

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

1801

#16

MAIL ROOM JUN 12 1995 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: 1806

Examiner: D. Adams

1802

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 9, 1995
(Date of Deposit)

Wendy Lee
Name of Depositing Party

Signature of Depositing Party

June 9, 1995
Date of Signature

AMENDMENT TRANSMITTAL

RECEIVED AUG 7 1995

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated as shown below.

	Claims Remaining After Amendment		Highest No. Previously Paid For	Present Extra	Rate	Additional Fee(s)
Total	24	Minus	23	= 1	x 22 =	$ 22.00
Indep.	6	Minus	10	= 0	x 76 =	$ 0
___ First Presentation of Multiple Dependent Claim					+ 240 =	$ 0
					TOTAL	**$ 22.00**

___ No additional fee is required.

__X__ The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $22.00. **A duplicate copy of this transmittal is enclosed.**

__X__ 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.

01 FC:180 102 07-0630 07/12/95 08146206 22.00CH 709P1

Date: June 9, 1995

By: _____
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

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: 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
June 9, 1995
(Date of Deposit)
Wendy Lee
Name of Depositing Party
[signature]
Signature of Depositing Party
June 9, 1995
Date of Signature

RECEIVED
AUG 7 1995

PETITION AND FEE FOR EXTENSION OF TIME (37 CFR 1.136(a))

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Applicant petitions the Commissioner of Patents and Trademarks to extend the time for response to the Office Action dated 12/9/94 for three month(s) from 3/9/95 to 6/9/95. The extended time for response does not exceed the statutory period.

Please charge Deposit Account No. 07-0630 in the amount of $870.00 to cover the cost of the extension. Any deficiency or overpayment should be charged or credited to this deposit account. A duplicate of this sheet is enclosed.

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: June 9, 1995

By: [signature]
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

250 TL 07-0630 07/12/95 08146206
25181 117 870.00CH 709P1

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: 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

June 9, 1995
(Date of Deposit)

Wendy Lee
Name of Depositing Party

Wendy Lee
Signature of Depositing Party

June 9, 1995
Date of Signature

AMENDMENT UNDER 37 C.F.R. §1.111

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

This amendment is responsive to the Office Action dated 12/9/94. Attached is a petition and petition fee for a three-month extension of time making this response timely filed on or before 6/9/95. Please amend the application as follows:

IN THE SPECIFICATION:

On page 1, beneath the title and before the subheading "Field of the Invention", please insert the following:

--Cross References

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

On page 65, line 5, change "Relative" to read --Relative cell proliferation--;
line 6, delete "cell";
line 8, delete "proliferation‡";
line 11, delete "407" and insert --4.7 101--;

92

line 12, delete "466" and insert --4.4 66--;
line 13, delete "0.80" and insert --0.82 56--;
line 14, delete "148" and insert --1.1 48--;
line 15, delete "0.22" and insert --0.22 51--;
line 16, delete "0.63" and insert --0.62 53--;
line 17, delete "0.50" and insert --0.10 54--; and
line 18, delete "0.30" and insert --0.30 37--.

IN THE CLAIMS:

Please cancel claims 13 and 14 without prejudice.

1. (Amended) A method for making a humanized antibody comprising amino acid sequences 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] Complementary Determining Region (CDR) amino acid sequences in the import variable domain and the consensus human [amino] variable domain [sequences];

{c}[.] substituting an import CDR amino acid sequence for the corresponding consensus human CDR amino acid sequence;

{d}[.] aligning the amino acid sequences of a Framework Region (FR) of the import [antibody] variable domain and [the] a corresponding FR of the consensus [antibody] human variable domain;

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

{f}[.] determining if the non-homologous import [amino acid] FR 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 by affecting the proximity or orientation of the V_L and V_H regions with respect to one another; and

{g}[.] for any non-homologous import [antibody amino acid] FR 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. (Amended) The method of claim 1, having an additional step of determining [if] whether any such non-homologous import residue[s are] is exposed on the surface of the consensus human variable domain or buried within it, and if the non-homologous import residue is exposed, retaining the corresponding consensus residue.

3. (Amended) 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] human variable domain.

4. (Amended) The method of claim 1 or 19, having the additional steps of searching the consensus human variable domain sequence for glycosylation sites which are not present at the corresponding amino acid in the import variable domain sequence, and if [the] any such glycosylation site is not present in the import variable domain sequence, substituting the import amino acid residue[s] for the amino acid residue[s] comprising the consensus glycosylation site.

5. (Amended) The method of claim 1 or 19, having [an] the additional steps if [which comprises] aligning the import [antibody] FR sequence and consensus [antibody] FR sequence[s], identifying import [antibody] FR residues which are non-homologous [with] to the aligned consensus FR [sequence] residues, 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. (Amended) The method of claim 1, wherein the corresponding consensus FR [antibody] residues substituted in step (g) 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, and 78H[, 91H, 92H, 93H, and 103H].

7. (Amended) A method comprising providing at least a portion of an import, non-human [antibody] variable domain amino acid sequence having a Complementary Determining Region (CDR) and a Framework Region (FR), obtaining the amino acid sequence of at least a portion of a consensus human [antibody] variable domain of a human immunoglobulin subgroup 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 a[n] non-human 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, and 78H[, 91H, 92H, 93H, and 103H].

In claim 8, line 2, please replace "antibody" with --variable domain--.
In claim 9, line 1, please delete "~~or 7~~".

10. (Amended) A humanized antibody variable domain having a non-human Complementary Determining Region (CDR) incorporated into a consensus human variable domain [a human antibody variable domain], wherein [the improvement comprises substituting an] a human amino acid residue [for the human residue] has been substituted by a non-human amino acid residue at a site 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, and 78H[, 91H, 92H, 93H, and 103H].

In claim 12, line 1, please replace "FR" with --Framework Region (FR)--.

15. (Amended) A method for engineering a humanized antibody comprising introducing amino acid residues from a[n] non-human, import [antibody] variable domain into [an amino acid sequence representing a] consensus [of mammalian antibody] human variable domain [sequences] of a human immunoglobulin subgroup.

19. (Amended) A method for making a humanized antibody comprising amino acid sequences 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] Complementary Determining Region (CDR) amino acid sequences in the import variable domain and the consensus human [amino] variable domain [sequences];

[(c)][.] substituting an import CDR amino acid sequence for the corresponding consensus human CDR amino acid sequence;

{d}[.] aligning the amino acid sequences of a Framework Region (FR) of the import [antibody] variable domain and [the] a corresponding FR of the consensus [antibody] human variable domain;

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

{f}[.] determining if the non-homologous import [amino acid] FR 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 by affecting the proximity or orientation of the V_L and V_H regions with respect to one another;

{g}[.] for any non-homologous import [antibody amino acid] FR 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] whether any such non-homologous import residue is exposed on the surface of the consensus human variable domain or buried within it, and if the non-homologous import residue is exposed, retaining the corresponding consensus residue.

Please add the following claims:

--20. The method of claim 1 wherein step (g) is followed by a step wherein the humanized antibody is prepared which has a variable domain having amino acid sequences determined in steps (a)-(g)--

--21. The method of claim 1 wherein the consensus human variable domain is of a human immunoglobulin subgroup.--

--22. The method of claim 19 wherein the consensus human variable domain is of a human immunoglobulin subgroup.--

--23. A humanized antibody comprising a consensus human variable domain of a human immunoglobulin subgroup wherein the amino acid residues forming the Complementary Determining Regions (CDRs) thereof comprise non-human import antibody amino acid residues.--

--24. The humanized antibody of claim 23 further comprising a Framework Region (FR) residue of the non-human import antibody, wherein the FR residue either:

(a) non-covalently binds antigen directly;

(b) interacts with a CDR;

(c) comprises a glycosylation site which affects the antigen binding or affinity of the antibody; or

(d) participates in the V_L - V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another.--

--25. The humanized antibody of claim 24 comprising more than one FR residue of the non-human import antibody.--

--26. The humanized antibody of claim 25 comprising from about 1 to about 7 FR residues of the non-human import antibody.--

REMARKS

The specification has been amended to correct obvious typographical errors in Table 3 on page 65. It is clear that the last two columns of Table 3 were inadvertently superimposed and the amendment to the specification serves merely to correct these errors. Please refer to Table 1 of Carter *et al., Proc. Natl. Acad. Sci.,* **89,** (1992), of record, which shows the correct Kd and Relative Cell Proliferation values of the variants described in Table 3 of the instant application. Applicants respectfully request that the specification be amended to correct the typographical errors discussed above.

The claims have been revised and additional claims added with specification support for the claim revisions being found at least as follows:

Claim	Wording	Specification Support
1, step (f)(3) 19, step (f)(3)	"by affecting....one another"	Page 11, lines 37-38
7, 15, 23	"of a human immunoglobulin subgroup"	Page 8, lines 27-29 Page 14, lines 3-4
21, 22	Entire Claim	
10, 23	"consensus human variable domain"	Claim 1 originally filed
20	Entire Claim	Page 1, line 6

23	"wherein the....import antibody amino acid residues"	Page 9, lines 32-38
24, 25	Entire Claim	Claims 1 and 3 originally filed
26	Entire Claim	See below

Claim 26 refers to the number of non-human import FR residues substituted into the humanized antibodies described in the examples (*i.e.* from about 1 to about 7 residues). In Example 1, 1-7 residues in the FR region were replaced with non-human import residues (see Table 3 on page 65). Murine residues are shown in three letter amino acid code (see lines 20-21 on page 65). Example 3 refers to replacement of 4 of the consensus FR residues with murine import residues (see Fig 5). Replacement residues are indicated with a "#" and residues in the CDRs are indicated by a line and/or carets.

The other claim revisions are clerical in nature. Following entry of this amendment, claims 1-12, 15 and 19-26 will be pending in this case.

Applicants note that the restriction requirement has been made final. Accordingly, claims 13 and 14 have been cancelled without prejudice to file a continuing application directed thereto.

Applicants note that claims 1-12 and 15 are currently under consideration. It should be noted that independent claim 19 (and claims 3, 4 and 5 which depend thereon) are also in this case, having been introduced in the amendment (dated June 12, 1993) to the PCT application on which this application is based. See the International Preliminary Examination Report dated September 20, 1993. Applicants ask that this claim also be considered in the prosecution of the instant application.

Formality Matters

The Examiner asserts that the 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 USC §120 which discloses and claims subject matter in addition to that disclosed in the prior copending application acknowledged the duty to disclose material information as defined in 37 CFR §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.

Applicants refer to the Combined Declaration and Power of Attorney submitted November 17, 1993. Since the declaration meets all the requirements of 37 CFR §1.63, applicants submit that a new declaration pursuant to 37 CFR §1.67(a) [see also MPEP 602.01 and 602.02] is not required. In particular, the last paragraph on page 1 of the declaration meets the requirements of 37 CFR §1.63(d). Accordingly, applicants request that the objection to the declaration be reconsidered and withdrawn.

The Examiner has objected to the drawings. Applicants ask that this matter be held in abeyance until the application is allowed.

The specification has been updated to refer to continuing data as proposed under item #29 in the Office Action.

The Rejection Under 35 USC §112, First Paragraph

The specification has been objected to and claims 1-12 and 15 rejected under 35 USC §112, first paragraph as allegedly failing to adequately teach how to use the claimed antibody or antibody produced by the claimed methods. The Examiner acknowledges that the exemplary antibody 4D5 does have a diagnostic utility for the detection of $p185^{HER2}$. However, the Examiner is of the opinion that it is unclear whether any other antibody will have a diagnostic or therapeutic utility. The Examiner believes that determining which other antibodies are useful would be an unpredictable event and would require undue experimentation for an ordinarily skilled person.

Applicants submit that the specification does enable the instantly claimed invention. This application discloses and claims a unique method for antibody humanization which can be used to humanize any antibody of interest. The instantly claimed humanization technique has been successfully used to humanize several different non-human antibodies including anti-HER2 (see Example 1); anti-CD3 (see Example 3); anti-CD18 (see Example 4); and anti-IgE (see Presta *et al.*, *J. Immunol.* **151**:2623-2632 [1993], copy attached). These antibodies had known diagnostic and/or therapeutic uses at the priority date of the instant application. For example, humanized anti-HER2 could be used for clinical intervention in and imaging of carcinomas in which $p185^{HER2}$ is overexpressed (see page 4, lines 20-28 of the application); humanized anti-CD3 antibodies could be used to detect CD3 in biological samples (*e.g.* to detect $CD3^+$ CTL; see page 69, line 22 of the application) or for making bispecific antibodies such as the anti-HER2/anti-CD3 bispecific antibody for tumor immunotherapy (see page 70, lines 23-38 of the application); anti-CD18 antibodies could be used for detecting the CD18 antigen in biological specimens and for indications such as reducing inflammation associated with meningitis or encephalitis (see U.S. Patent 5,147,637, copy attached), for example; anti-IgE could be used for detecting IgE and for treating allergy as described in Presta *et al.*, *supra*. In addition to these antibodies, the application refers to many other antibodies available at the priority date which were known to have diagnostic and/or therapeutic uses. These antibodies presented potential candidates for humanization using the procedures disclosed and claimed. Examples are provided in the background section of the application. See, for example, Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, MD, (1987); U.S. patent No. 4,816,567; Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* **81**:6851-6855 (1984); Boulianne *et al.*, *Nature* **312**:643-646 (1984); Neuberger *et al.*, *Nature* **314**:268-270 (1985); Brüggemann *et al.*, *J. Exp. Med.* **166**:1351-1361 (1987); Riechmann *et al.*, *Nature* **332**:323-327 (1988); Love *et al.*, *Methods in Enzymology* **178**:515-527 (1989); Bindon *et al.*, *J. Exp. Med.* **168**:127-142 (1988); Jaffers *et al.*, *Transplantation* **41**:572-578 (1986); Jones *et al.*, *Nature* **321**:522-525 (1986); Verhoeyen, M. *et al.*, *Science* **239**:1534-1536

(1988); Hale *et al., Lancet* **i**:1394-1399 (1988); Queen *et al., Proc. Natl. Acad. Sci. USA* **86**:10029-10033 (1989); Co *et al., Proc. Natl. Acad. Sci. USA* **88**:2869-2873 (1991); Gorman *et al., Proc. Natl. Acad. Sci. USA* **88**:4181-4185 (1991); Daugherty *et al., Nucleic Acids Research* **19(9)**:2471-2476 (1991); Brown *et al., Proc. Natl. Acad. Sci. USA* **88**:2663-2667 (1991); and Junghans *et al., Cancer Research* **50**:1495-1502 (1990), all of record. Therefore, any of the antibodies described in these references could have been chosen to be humanized using the techniques described in the instant application. In addition, an antibody to the antigens described in these references or other antigens of interest could have been generated using the techniques for making antibodies described on pages 27-29 of the application, for example. Therapeutic and diagnostic uses for the humanized antibodies were also taught on, *e.g.*, pages 50-55 of the application.

In addition to the numerous examples of antibodies which were specifically disclosed in the application, the skilled practitioner at the priority date would have had many, many other antibodies with established uses (including diagnostic and therapeutic uses) to choose from. To demonstrate this, several review articles are attached which show that antibodies which were used (a) as probes for oncogene products; (b) as tools in genetic studies on carbohydrate blood group antigens; (c) for diagnosis and therapy of lymphoproliferative diseases; (d) in the diagnosis and treatment of bacterial infections; (e) in the diagnosis and prognosis of breast cancer; (f) in the flow cytometric analysis of benign and malignant cells; and (g) as proliferation markers (*e.g.* Ki-67) for immunohistological diagnostic and prognostic evaluation of human malignancies, were available at the priority date which could have been humanized using the instantly claimed method. See Niman, *Immunodiagnosis of Cancer*, Second Edition, pp. 189-204 (1990); Watkins *et al., Journal of Immunogenetics* **17**:259-276 (1990); Campana *et al., The Turkish Journal of Pediatrics* **32**:143-151 (1990); Verhoef and Torensma, *Eur. J. Clin. Microbiol. Infect. Dis.* **9(4)**:247-250 (1990); Ellis *et al., Pathology Annual* **25**:193-235 (1990); Beck *et al., Cancer Biology* **1**:181-188 (1990); and Gerdes, *Cancer Biology* **1**:199-206 (1990), copies attached. Once the method of humanization disclosed in the instant application was discovered, it would have been routine to select any one of these antibodies and humanize them using the disclosed procedures. Therefore, applicants submit that it would have been clear to the skilled artisan that many antibodies other than anti-HER2 were available which had diagnostic and/or therapeutic utilities. Applicants further submit that determining which other antibodies would have been useful at the priority date would not have been an unpredictable event and would not have required undue experimentation for an ordinarily skilled person.

Accordingly, applicants ask that this rejection under 35 USC §112, second paragraph be reconsidered and withdrawn.

The Rejection Under 35 USC §103 - Winter, Queen *et al.* and Riechmann *et al.*

Claims 1, 2, 4-12 and 15 are rejected under 35 USC §103 as being unpatentable over EP239,400 (Winter); Riechmann *et al.* Nature: **332**: 323-327 (1988); and Queen *et al.* PNAS, USA **86**: 10029-10033 (1989). Applicants traverse this rejection as it may apply to the claims as amended herein.

EP239,400 describes a procedure for partial antibody "humanization" wherein the FR residues of the heavy chain of the engineered antibody are provided by the framework region of an individual human antibody V_H. In particular, the heavy chain framework region of the humanized B1-8 antibody (*i.e.* HuV_{NP}) described in Example 1 and the humanized anti-lysozyme antibody D1.3 described in Example 2 was derived from the human myeloma heavy chain NEWM (see page 17, lines 1-2 and lines 9-10 on page 26). The $NEWV_H$ framework region was chosen because the crystallographic structure thereof was known. See page 17, lines 2-3 of EP239,400. The light chains of the B1-8 and D1•3 antibodies were never humanized. Furthermore, only the CDRs were transferred; none of the non-human FR residues were incorporated into the engineered molecule. EP239,400 briefly mentions further work with the antibody CAMPATH-1 (see pages 30-31), but fails to describe in detail how this antibody was humanized. The detailed description of the "CAMPATH-1" work appears to be described in Riechmann *et al.* Using the same strategy as disclosed in EP239,400, Riechmann and his colleagues made a humanized heavy-chain variable domain which had the framework regions of human NEW alternating with the CDRs of rat YTH 34.5HL anti-CAMPATH-1 antibody. Thus, the same heavy chain framework region as disclosed in EP239,400 was used once again. The rationale for this was that the crystallographic structure of NEW was available (see page 325, second to last paragraph of Riechmann *et al.*). For humanization of the light chain of rat YTH 34.5HL, the human REI light chain variable domain was used, as the human NEW light chain region could not be used (because there is a deletion at the beginning of the third framework region of NEW; see page 325, second to last paragraph of Riechmann *et al.*). Also, a crystallographic structure for REI was available. Thus, Riechmann *et al.* used FR residues from a single antibody for humanizing a non-human antibody variable domain. Riechmann *et al.* describe mutating one or two FR residues in order "to restore the packing of the loop" (see page 326, column 1).

Queen *et al.* describe the methods they employed for humanizing their anti-Tac monoclonal antibody which binds to the p55 chain of the human interleukin 2 receptor. As mentioned in the abstract of this paper, the "human framework regions were chosen to maximize homology with the anti-Tac antibody sequence". Queen *et al.* reasoned that the more homologous the human antibody was to the original anti-Tac antibody, the less likely would combining the anti-Tac CDRs with the human FR be to introduce distortions into the CDRs. See page 10031, column 2, paragraph 2 of Queen *et al.* Queen *et al.* further reiterate this in the summary on page 10033 where they state that "the human framework was chosen to be as homologous as possible to the original mouse antibody to reduce any

deformation of the mouse CDRs". Thus, based on a comparison of the anti-Tac heavy chain sequence to all human heavy chain sequences in the National Biomedical Research Foundation Protein Identification Resource database, the heavy chain V region of the human Eu antibody was selected. Because no one human light chain V region was especially homologous to the anti-Tac light chain, the Eu light chain was also selected to provide the framework residues for the light chain of the humanized antibody. Accordingly, the framework regions of the humanized antibody described by Queen *et al.* were derived from a single antibody. Queen *et al.* transferred a number of the murine FR residues into the humanized antibody (two in the V_L and nine in the V_H; see Fig. 2 of this reference). These transferred residues were thought to be close enough to the CDRs to either influence their conformation or interact directly with antigen (see page 10031, column 2, paragraph 3). It was thought that this transfer of FR residues would better preserve the precise structure of the CDRs at the cost of possibly making the humanized antibody slightly less "human". Queen *et al.* also noted that a given human variable domain will contain exceptional FR amino acids which are atypical of other human V regions. The human Eu antibody had seven such residues in the heavy chain and two in the light chain. Because the murine antibody had a residue much more typical of human sequences, the murine residues were retained at these sites rather than the Eu residue.

The instantly claimed invention differs from the teachings of each of the above-mentioned references in that it provides a method for humanization and humanized antibodies wherein the framework regions of the humanized antibodies are essentially formed by a "consensus human variable domain", *i.e.*, an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all human immunoglobulins of any particular subclass or subunit structure (see page 13, lines 20-22 of the application). Preferably, the consensus is from one of the "human immunoglobulin subgroups" described by Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda MD (1987) (*e.g.*, V_L κ subgroup I and V_H subgroup III). See page 14, first paragraph of the application. The instant application demonstrates, for the first time, that a number of non-human antibodies can be humanized using such a consensus human variable domain to provide the framework regions of the antibody. Applicants submit that the use of such a consensus sequence for humanizing non-human antibodies was not disclosed or alluded to by the cited references. Accordingly, a *prima facie* case of obviousness has not been established by the Office.

In addition, the prior art taught away from the claimed invention. EP239,400 and Riechmann *et al.* taught that a framework region of an individual antibody should be used for humanization, especially where a crystallographic structure of the chosen antibody was available. On the contrary, crystal structures of consensus human variable domains as claimed in the instant application were not available. Therefore, the method for humanization claimed in the above application diverged from that taught by EP239,400 and Riechmann *et al.*

Queen *et al.* also taught that the FR residues of the humanized antibody should be provided by an individual antibody (*i.e.* the Eu antibody). Furthermore, Queen *et al.* taught that the sequence used for humanization should be as homologous as possible to the non-human sequence to be humanized in order to reduce the likelihood of introducing distortions into the CDRs. Therefore, according to the teachings of Queen *et al.* framework region sequences needed to be tailored to each non-human antibody to be humanized. Because Queen *et al.* used the human Eu antibody sequence, they found that they needed to replace "atypical" residues from the human sequence with the corresponding murine residues (where the murine residues were more typical). See page 10032, column 1, paragraph 1 of Queen *et al.* The approach adopted by Queen *et al.* was also followed by Co *et al.*, PNAS USA, **88**:2869-2873 (1991), of record. It is apparent that Co *et al.* felt it was necessary to follow the strategy of Queen *et al.* if one considers the statements made on 2871 (column 1) of their paper. In particular, Co *et al.* say "To retain high binding affinity in the humanized antibodies, the general procedures of Queen *et al.*(15) were followed. First, a human antibody variable domain with maximal homology to the mouse antibody is selected to provide the framework sequence for humanization of the mouse antibody. Normally the heavy chain and light chain from the same human antibody are chosen so as to reduce the possibility of incompatibility in the assembly of the two chains". The humanization technique of Queen *et al.* and Co *et al.* has now been coined the "best-fit" method for humanization insofar as it relies on selecting an individual human antibody which is as homologous in sequence as possible to the non-human sequence which is to be humanized. Furthermore, these references teach that the heavy chain and light chain used for humanization should be derived from the same human antibody.

On the other hand, the instantly claimed invention constitutes a bold new approach to humanization that does not rely on a high degree of sequence homology between the human and non-human sequences and does not require the existence of a crystallographic structure of the human antibody; the framework regions of the antibodies humanized using the instantly claimed techniques are consensus human variable domain sequences. Applicants submit that the skilled practitioner would have had no motivation to use consensus sequences to form the framework regions of humanized antibodies at the priority date, since the prior art taught that the framework regions should be provided by individual human antibody sequences. Furthermore, the skilled artisan would have been motivated not to use a consensus human variable domain, as the Queen *et al.* and Co *et al.* references taught that the framework region sequences should be chosen based on their sequence homology to the non-human antibody. The instantly claimed invention shows that, contrary to what would have been expected, the claimed consensus sequences can be used for humanization of many different non-human antibodies. This is a significant finding for at least the following two reasons.

First, one must consider why antibodies are humanized. Antibody humanization provides a means for reducing immunogenicity, tailoring effector functions and increasing serum half-life. The

instantly claimed invention provides an improvement in relation to the first of these, *i.e.,* reducing immunogenicity. By using a consensus sequence, which is a sequence comprising the most commonly occurring amino acid at each site in the heavy or light chain, the likelihood that an "atypical" amino acid residue may be present in the framework of the humanized antibody is reduced. Such atypical framework region residues are thought to be detrimental because the human immune system may recognize these as foreign. Thus, the instantly claimed invention obviates the need to replace atypical human residues as taught by Queen *et al.* Therefore, the instantly claimed invention also constitutes a "minimalistic" approach wherein as few non-human residues as possible are incorporated into the humanized antibody, thus reducing the potential immunogenicity of the humanized antibody (see 75, lines 9-11 of the instant application).

The other advantage of the instantly claimed invention is that applicants have shown that a selected V_H consensus sequence and selected V_L consensus sequence can be used to humanize many different non-human antibodies including anti-HER2 (see Example 1); anti-CD3 (see Example 3); anti-CD18 (see Example 4); and anti-IgE (see Presta *et al., supra*). In particular, applicants have seen that humanized anti-HER2 and humanized anti-IgE do not lead to detectable immunogenic responses upon administration to humans. Thus, the claimed method is clearly useful for the production of humanized antibodies with reduced immunogenicity. The techniques advocated by the prior art, especially Queen *et al.* and Co *et al.,* would not allow for this flexibility, since for each new non-human antibody to be humanized, a human antibody sequence with high homology thereto must be used.

To further emphasize the differences between the approaches of the cited references (where FRs from individual human antibodies are used) and the consensus approach which is instantly claimed, applicants refer to the following references. In particular, Sims *et al., J. Immunol.* **151(4)**:2296-2308 (1993), copy attached, used the "best-fit" method to humanize their anti-CD18 antibody. See column 2, paragraph 3 on page 2302. Kolbinger *et al.* further contrast the differences between the individual antibody approach and the consensus approach which is claimed in the above application. See Kolbinger *et al., Protein Engineering* **6**:971-980 (1983) (copy attached). As mentioned in the abstract of Kolbinger *et al.* "Two approaches to the selection of human FRs were tested: (i) selection from human consensus sequences and (ii) selection from individual human antibodies". Kolbinger *et al.* used the consensus sequences for human κ V_L subgroup III and human V_H subgroup I (see Figures 2 and 3) for one version of a humanized antibody. The other humanized antibody was made using the "best-fit" method (see page 977, column 1). In the best-fit method, the V_L of the human antibody KAF and the V_H of the human antibody HAY were used for humanization (see Figures 2 and 3 of Kolbinger *et al.*). Thus, those skilled in the art have acknowledged that the techniques of the prior art and the technique of the instant applicant are certainly different. Accordingly, applicants believe that the invention recited in the claims at issue is clearly non-obvious over the references and the rejection should therefore be reconsidered and withdrawn.

Not only do the cited references fail to disclose or suggest the use of the consensus human antibody variable domain for humanization, but they also fail to address other aspects of the instantly claimed invention. In particular, the references fail to describe steps (f) and (g) of claims 1 and 19 of the instant application. These steps instruct the practitioner concerning selection of human FR residues to be replaced with corresponding non-human residues. In particular, non-homologous non-human FR amino acid residue(s) which are expected to non-covalently bind antigen directly, interact with a CDR, or participate in the V_L - V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another are introduced into the consensus FR. The cited references fail to enable these steps. In particular, EP239,400 does not elaborate in sufficient detail how one would go about selecting non-human FR residues to be incorporated into the humanized antibody. Significantly, no non-human FR residues were transferred in the examples of EP239,400. While Riechmann *et al.* made one and two FR residue mutations to "restore the packing of the loop", this reference fails to describe each of the types of non-homologous residue identified in items (1)-(3) of step (f) of claims 1 and 19 of the instant application. Queen *et al.* also fail to describe the transfer of non-homologous residues which participate in the V_L - V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another (see step (f)(3) of claims 1 and 19 of the instant application). Hence, the invention recited in claims 1 and 19 is clearly not obvious over the references.

The instantly claimed invention has other novel and non-obvious features. For example, claim 2 and step (h) of claim 19 of the instant application involve retaining consensus residues, where the corresponding non-homologous import residues are exposed of the surface of the consensus human variable domain. The cited references fail to describe anywhere such a step. Claim 4 involves replacing consensus glycosylation sites which are not present in the import sequence with the corresponding non-human residue. The references are silent as to such a step. Similarly, the references fail to describe the additional step of claim 5 of the instant application. Also, the FR residues which can be substituted and are listed in claims 6, 7 and 10 as revised herein are not disclosed or alluded to in the cited references. Thus, applicants submit that the invention recited in the claims of the instant application is clearly non-obvious over the cited references.

Accordingly, applicants request that the above section 103 rejection be reconsidered and withdrawn.

The Rejection Under 35 USC §103 - *In re* Durden

Claims 1, 2, 4-12 and 15 are rejected under 35 USC §103 as being unpatentable over EP239,400 (Winter); Riechmann *et al.* Nature: **332**: 323-327 (1988); and Queen *et al.* PNAS, USA **86**: 10029-10033 (1989) in view of In re Durden 226 USPQ 359 (Fed. Cir. 1985).

The Examiner states that the claimed methods for producing humanized antibodies and humanized antibodies do not appear to differ from what was disclosed in the references. For the

reasons given in the previous section, applicants submit that the instantly claimed methods for humanization and the humanized antibodies are clearly different from what was disclosed in the cited reference, especially with respect to the consensus human variable domain forming the FR of the humanized antibody. Therefore, applicants request that this rejection be reconsidered and withdrawn.

The Rejection Under 35 USC §103 - Claim 3

Claim 3 is rejected under 35 USC §103 as being unpatentable over EP 239,400 (Winter); Riechmann *et al.* Nature: **332**: 323-327 (1988); and Queen *et al.* PNAS, USA **86**: 10029-10033 (1989) as applied to claims 1, 2, 4-12 and 15 and further in view Roitt *et al.,* Immunology Gower Medical Publishing Ltd., London, England, pg. 5.5 (1985). It is the Examiner's position that, since Roitt *et al.* allegedly teaches that antibodies contain carbohydrate residues in the variable region, a person skilled in the art would realize that carbohydrate residues can produce stearic modifications in the folding characteristics of polypeptides. The Examiner concludes that it would have been *prima facie* obvious to carry out the step recited in claim 3.

Applicants submit that the claim 3 is clearly not obvious in light of the cited references. The three primary references have been discussed above. Roitt *et al.* merely shows that IgA1 immunoglobulins may possibly have carbohydrate units in their variable domains. No such carbohydrate or oligosaccharide units are depicted in the diagrams of IgD and IgE variable domains in this reference. This reference is not concerned with antibody humanization, much less the use of a consensus human variable domain for humanization or how to deal with glycosylation sites in humanization. Since claim 3 depends on claim 1 which specifies the use of a consensus human variable domain, and since neither the primary references nor Roitt *et al.* disclose or allude to the use of such a consensus sequence, claim 3 must also be nonobvious over the references. Furthermore, the primary references and Roitt *et al.* fail to address how one would deal with glycosylation sites in the context of humanization. In fact, 4D5 referred to in Example 1 is fairly unusual in that it has a glycosylation site in its variable region (*i.e.* residue number 65 of the light chain). Thus, as far as applicants are aware, the instant application teaches, for the first time, how to deal with glycosylation sites in antibody humanization.

Accordingly, applicants conclude that claim 3 is clearly not obvious in light of the references cited and therefore ask that the §103 rejection be withdrawn.

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: June 9, 1995 By: _____
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

Enclosures

U.S. Patent No. 5,147,637
Presta *et al., J. Immunol.* **151**:2623-2632 (1993)
Niman, *Immunodiagnosis of Cancer*, Second Edition, pp. 189-204 (1990)
Watkins *et al., Journal of Immunogenetics* **17**:259-276 (1990)
Campana *et al., The Turkish Journal of Pediatrics* **32**:143-151 (1990)
Verhoef and Torensma, *Eur. J. Clin. Microbiol. Infect. Dis.* **9(4)**:247-250 (1990)
Ellis *et al., Pathology Annual* **25**:193-235 (1990)
Beck *et al., Cancer Biology* **1**:181-188 (1990)
Gerdes, *Cancer Biology* **1**:199-206 (1990)
Sims *et al., J. Immunol.* **151(4)**:2296-2308 (1993)
Kolbinger *et al., Protein Engineering* **6**:971-980 (1983)

UNITED STATES PATENT AND TRADEMARK OFFICE
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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.

This document constitutes proof of such recognition. The original of this document is on file in the Office of Enrollment and Discipline of the U.S. Patent and Trademark Office.

EXPIRES: DECEMBER 9, 1995

Cameron Weiffenbach, Director
Office of Enrollment and Discipline

PATENT DOCKET 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: 1806

Examiner: D. Adams

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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
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SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

BOX DD
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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.** The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $130.00 to cover the cost of this Information Disclosure Statement. Any deficiency or overpayment should be charged or credited to this deposit account. **A duplicate of this sheet is enclosed**.

[If either of boxes (d) or (e) is checked above, the following "certification" under 37 CFR §1.97(e) may need to be completed.] The undersigned certifies that:

[] Each item of information contained in the information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement.

[] No item of information contained in this information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application or, to the knowledge of the undersigned after making reasonable inquiry, was known to any individual designated in 37 CFR §1.56(c) more than three months prior to the filing of this information disclosure statement.

A list of the patent(s) or publication(s) is set forth on the attached Form PTO-1449 (Modified).

A copy of the items on PTO-1449 is supplied herewith:

[X] each [] none [] only those listed below:

Those patent(s) or publication(s) which are marked with an asterisk (*) in the attached PTO-1449 form are not supplied because they were previously cited by or submitted to the Office in a prior application Serial No. , filed and relied upon in this application for an earlier filing date under 35 USC §120.

A concise explanation of relevance of the items listed on PTO-1449 is:

[X] not given

[] given for each listed item

[] given for only non-English language listed item(s) [Required]

[] in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references.

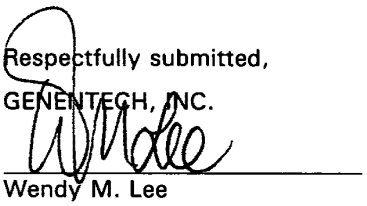
The Examiner is reminded that a "concise explanation of the relevance" of the submitted prior art "may be nothing more than identification of the particular figure or paragraph of the patent or publication which has some relation to the claimed invention," MPEP §609.

While the information and references disclosed in this Information Disclosure Statement may be "material" pursuant to 37 CFR §1.56, it is not intended to constitute an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

In accordance with 37 CFR §1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR §1.56(a) exists. It is submitted that the Information Disclosure Statement is in compliance with 37 CFR §1.98 and MPEP §609 and the Examiner is respectfully requested to consider the listed references.

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: August 1, 1995 By: 
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

Paper # 15

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(71) Applicant *(for all designated States except US)*: SCOTGEN LIMITED [GB/GB]; Queen's House, 2 Holly Road, Twickenham, Middlesex TW1 4EG (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : HARRIS, William, J. [GB/GB]; 3 Caesar Avenue, Carnoustie, Angus DD7 6DR (GB). TEMPEST, Philip, R. [GB/GB]; 63 Brighton Place, Aberdeen AB1 6RT (GB). TAYLOR, Geraldine [GB/GB]; Robinsgrove, Wallingford Road, Compton, Berkshire RG16 0PT (GB).

(74) Agent: VALENTINE, Jill, Barbara; SmithKline Beecham, Great Burgh, Yew Tree Bottom Road, Epsom, Surrey KT18 5XQ (GB).

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(57) Abstract

Altered antibodies in which at least parts of the complementary determining regions (CDRs) in the light and/or heavy variable domains of an acceptor monoclonal antibody have been replaced by analagous parts of CDRs from one or more donor monoclonal antibodies, and in which there has been minimal alteration of the acceptor monoclonal antibody light and/or heavy variable domain framework region in order to retain donor monoclonal antibody binding specificity, wherein such donor antibodies have specificity for microorganisms, in particular specificity for respiratory syncytial virus (RSV); a process for preparing such altered antibodies; a pharmaceutical composition comprising a therapeutic, non-toxic amount of such altered antibodies and a pharmaceutically acceptable carrier or diluent; a method of prophylactically or therapeutically treating a microorganism-induced disease state in a human or animal in need thereof which comprises administering an effective amount of such altered antibodies to such human or animal; a specific epitope of the F protein of RSV; monoclonal antibodies directed against such epitope; Fab fragments of such monoclonal antibodies; a pharmaceutical composition comprising a therapeutic, non-toxic amount of such monoclonal antibodies or Fab fragments and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or therapeutically treating RSV infection in a human or animal in need thereof which comprises administering an effective amount of such monoclonal antibodies or Fab fragments to such human or animal.

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NOVEL ANTIBODIES FOR TREATMENT AND PREVENTION OF INFECTION IN ANIMALS AND MAN

BACKGROUND OF THE INVENTION

There has long been a need for effective agents for prevention and treatment of infection in animals and man. Typical methods comprise administration of chemical agents which inhibit the growth of microorganisms allowing the immune system to eradicate the infectious agent. Whilst natural and synthetic chemicals have been particularly effective as treatments for bacterial infection, the emergence of resistant strains has proved frequent and problematic. For viral infections, chemical agents have had limited effect and the severity of disease is usually correlated with immune system status.

For many years, the effectiveness of serum from immune individuals on prevention and treatment of infectious disease has been known. However, it is well known that the antibodies within human immune sera which are responsible for effective treatment, i.e., the neutralising antibody component, are only a very small fraction of the total sera antibody. Furthermore, the use of immune sera has been limited by low neutralising antibody levels, by the scarcity of immune donors, by the cost of treatment and more recently by the risk of adventitious spread of disease through microorganisms in donor sera.

The development of monoclonal antibody technology provided the means for development and production of pure murine monoclonal antibodies in large quantities from cell lines devoid of pathogenic microorganisms. With this technique it was possible to provide monoclonal antibodies which interacted with pathogenic organisms, some of which monoclonal antibodies could prevent the growth of the target microorganisms in infected mice. Unfortunately, it is not possible to predict from in vitro studies which antibodies will be most effective at in vivo killing of microorganisms. Many

monoclonal antibodies with high binding affinity for their target in an in vitro setting are not effective in vivo. In fact, in some cases where antibodies are effective at preventing growth of the microorganisms under laboratory conditions, they prove ineffective in the in vivo environment.

The impact and limitations of murine monoclonal antibodies for treatment of infectious disease is illustrated by the case of respiratory syncytial virus (RSV) infection. RSV is the major cause of lower respiratory tract infection in infants in the first year of life and a significant cause of respiratory disease in young cattle. In man, most attempts to vaccinate against RSV infection have failed, and treatment of RSV infection with chemical drugs such as ribavirin is only partially effective. Murine monoclonal antibodies specific for RSV have been shown to be effective in prevention and treatment of RSV in mice. However, the use of murine monoclonal antibodies for treatment and prevention of RSV in non-murine species is potentially limited by the immune response of these species to the "foreign" murine antibody, i.e., immune responses in humans against murine antibodies have been shown to both immunoglobulin constant and variable regions (human anti-mouse antibodies). Therefore, non-immunogenic variants of monoclonal antibodies where the immunoglobulin constant and variable regions contain amino acid sequences recognised as "self" by the RSV infected recipient are needed for effective prevention and treatment of RSV infection.

Recombinant DNA technology has provided the ability to alter antibodies in order to substitute specific immunoglobulin (Ig) regions from one species with regions from another. Patent Cooperation Treaty Patent Application No. PCT/GB85/00392 (Neuberger et al and Celltech Limited) describes a process whereby the complementary heavy and light chain variable domain of an Ig molecule from one species may be combined with the complementary heavy and light chain Ig constant domains from another species. This process may be used, for example, to alter murine monoclonal antibodies directed against a specific human disease. Such alteration is effected by substitution of the murine antibody constant region domains with human IgG constant region domains to create a "chimeric" antibody to

be potentially used for treatment of such human disease. However, such chimeric antibodies will still potentially elicit an immune response in humans against the murine (i.e, "foreign") variable regions.

British Patent Application Publication Number GB2188638A (Winter) describes a process whereby antibodies are altered by substitution of their complementarity determining regions (CDRs) from one species with those from another. This process may be used, for example, to substitute the CDRs from human heavy and light chain Ig variable region domains with alternative CDRs from murine variable region domains. These altered Ig variable regions may subsequently be combined with human Ig constant regions to create antibodies which are totally human in composition except for the subsitituted murine CDRs. Such murine CDR-substituted antibodies are likely to elicit a considerably reduced immune response in humans compared to chimeric antibodies because they contain considerably less murine components. However, as stated in British Patent Application Publication Number GB2188638A, merely replacing one or more CDRs with complementary CDRs from another species which are specific for a desired disease may not always result in an altered antibody which retains the antigen binding capacity of complementary CDRs. The British Patent Application proposes that by "routine experimentation or by trial and error", a functional altered antibody with antigen binding capacity may be obtained. However, no description of the nature of the routine experimentation or the trial and error process needed to obtain the desired antibody is provided, and there is a suggestion that successive replacements of CDRs from different sources should be attempted.

Examination of the three-dimensional structures of several IgGs has led to the conclusion that the Ig variable regions of heavy and light chains each comprise three looped structures (which include the CDRs) supported on a sheet-like structure termed the variable region framework. The predominant definition of what comprises a CDR and what comprises a framework is based upon amino acid sequences of a number of Igs.

In three dimensional configuration, the aforementioned loop structures and CDRs between a mouse and human antibody do not correspond exactly although there is considerable overlap. Therefore it appears that, in some cases, the transfer of antigen binding specificity by replacement of CDRs may require the additional replacement of residues adjacent to the defined CDRs. For example, it has been hypothesized that , in certain cases, variable region framework amino acid residues may be important in antigen binding through direct interaction with CDRs (See, Amit et al., Science, 233 (1986) pp 747-753; Queen et al., Proc. Natl. Acad. Sci., 86 (1989) pp10029-10033; and Protein Design Labs, Patent Cooperation Treaty Patent Application Publication Number WO9007861, published July 26, 1990). In the Queen et al. reference, the authors selected human variable regions for murine CDR-replacement on the basis of maximum homology to the murine variable region comprising the CDRs used for the replacement. In addition, on the basis of computer modelling, the Queen et al. authors utilized a human framework for CDR replacement which included several murine framework amino acids thought to interact with the murine CDRs. The resultant altered antibody, whilst retaining antigen binding capacity, contained additional murine framework amino acids. Such addtional murine framework amino acids might contribute to an enhanced immune response to the altered antibody in humans.

In addition, previous studies (see, e.g., Riechmann,et al., Nature, 332 (1988), p323-327) have demonstrated that the use of reshaping can be used to transfer in vitro high affinity binding from mouse to human antibodies, but it has not previously been shown that it is possible to provide the combination of properties required for preservation of effective prevention of growth of human respiratory syncytial virus (RSV) in vivo.

Therefore, there is a need for altered antibodies with minimal immunogenicity for the prevention and treatment of infectious disease. In addition, there is a need for a defined process to produce such altered antibodies without radical alteration of variable region frameworks and the associated effect on immunogenicity. The present invention provides altered antibodies for prevention and treatment of infectious disease and a

process for their production by introducing only critical variable region framework modifications.

RSV, which is in the genus Pneumovirus of the *Paramyxoviridae* family, is a major cause of lower respiratory tract infections in young children. Primary infection gives an incomplete immunity, and reinfection is frequently observed during childhood. The role of immune mechanisms in the human disease have not been clarified. Previous attempts to develop effective vaccines with attenuated or killed RSV have met with failure, i.e., not only were the children unprotected, but subsequent infections with RSV sometimes resulted in more severe diseases than in non-immunized controls. RSV infection is also a major cause of respiratory infection in young cattle.

Recently, certain immunological and molecular information has been obtained regarding the antigenic and functional properties of RSV proteins. The RSV fusion protein (F) and the RSV attachment protein (G) have been identified as the major viral antigens, and their genes have been cloned and sequenced. Two antigenically distinct subgroups of human RSV, designated A and B, have been described. The antigenic differences between A and B subgroups reside mainly on the RSV G protein. In contrast, the RSV F protein has a high degree of genetic and antigenic homology between the two subgroups, and various strains within these subgroups.

Monoclonal antibodies (mAbs) directed against both envelope glycoproteins (F and G) of RSV have been demonstrated to neutralize the virus. (See, Walsh & Hruska, J. Virology, 47, 171-177 (1983); and Walsh et al., J. Gen. Virology, 65, 761-767 (1984)). However, *in vitro* and *in vivo* studies with mAbs or with vaccinia virus recombinants expressing F protein indicated that this protein is the most important antigen in inducing cross-protective immunity. (See, Johnson et al., J. Virology, 61, 3163-3166 (1987); Olmsted et al., Proc. Nat. Acad. Sci., USA, 7462-7466 (1986); Wertz

et al., J. Virology, 61, 293-310 (1987); and Walsh et al., Infection and Immunity, 43, 756-758 (1984)). Several authors have identified different antigenic sites in the F protein and have shown that at least three of these antigenic sites are involved in neutralization. Two or three neutralizing epitopes have been located on the F protein in different ways. Using escape mutant viruses, Lopez et al., J. Virology, 64, 927-930 (1990) have shown that two amino acid residues (i.e., 262-Asn and 268-Asn) of the F_1 subunit of the F protein are essential for the integrity of a particular neutralizing epitope. Another highly conserved neutralizing epitope has been mapped with synthetic peptides to residues 221-Ile to 232-Glu of the F1 subunit of the F protein by Trudel et al., J. General Virology, 68, 2273-2280 (1987). Finally, a recent analysis by the Pepscan procedure identified an epitope at positions 483-Phe to 488-Phe of the F1 subunit of the F protein, which epitope could correspond to another neutralizing epitope. (See, Scopes et al., J. General Virology, 71, 53-59 (1990)).

There is a need for the development of new therapies for the treatment and prevention of RSV infection. A neutralizing and protective epitope of an RSV viral antigen could prove useful in the generation of monoclonal antibodies useful for the prophylaxis and/or treatment of RSV infection. The present invention provides such a novel epitope on the RSV F protein which is recognised by a neutralizing and protective antibody *in vivo*.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the DNA sequence and corresponding amino acid sequence of the RSV19 heavy chain variable region (VH). The CDR sequences are boxed. The first eight and last eleven amino acids, as underlined, correspond to sequences of the oligonucleotide primers used.

Figure 2 shows the DNA sequence and corresponding amino acid sequence of the RSV19 light chain variable region (VK). The CDR sequences are

boxed. The first eight and last six amino acids, as underlined, correspond to sequences of the oligonucleotide primers used.

Figure 3 shows the basic plasmid pHuRSV19VH comprising a human Ig heavy chain variable region framework and CDRs derived from mouse RSV19.

Figure 4 shows the basic plasmid pHuRSV19VK comprising a human Ig light chain variable region framework and CDRs derived from mouse RSV19.

Figure 5 shows the derived Ig variable region amino acid sequences encoded by RSV19VH, RSV19VK, pHuRSVVH and pHuRSV19VK, and derivations of pHuRSV19VH.

Figure 6 shows an ELISA analysis of the binding of HuRSV19VH/VK antibody and its derivative, HuRSV19VHFNS/VK, to RSV antigen.

Figure 6A shows that there is little if any difference between the ability of the RSV19 and HuRSV19VHFNS/HuRSV19VK antibodies to bind to intact, non-denatured RS virus.

Figure 7 shows that mAb RSV19 binds to two synthetic peptides consisting of, respectively, amino acid residues 417-432 and 422-438 of the F protein.

SUMMARY OF THE INVENTION

The present invention relates to altered antibodies in which at least parts of the complementarity determining regions (CDRs) in the light and/or heavy variable domains of an acceptor monoclonal antibody have been replaced by analagous parts of CDRs from one or more donor monoclonal antibodies, and in which there may or may not have been minimal alteration of the acceptor monoclonal antibody light and/or heavy variable domain framework region in order to retain donor monoclonal antibody binding

specificity, wherein such donor antibodies have specificity for microorganisms, in particular specificity for respiratory syncytial virus (RSV). The present invention also relates to a process for preparing such altered antibodies; a pharmaceutical composition comprising a therapeutic, non-toxic amount of such altered antibodies and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or therapeutically treating a microorganism-induced disease state in a human or animal in need thereof which comprises administering an effective amount of such altered antibodies to such human or animal. Preferably the altered antibodies of the invention will be produced by recombinant DNA technology. The altered antibody of the present invention may comprise a complete antibody molecule (having full length heavy and light chains) or any fragment thereof, such as the Fab or $(Fab')_2$ fragment, a light chain or heavy chain dimer, or any minimal recombinant fragment thereof such as an Fv or a SCA (single-chian antibody) or any other molecule with the same specificity as the altered antibody of the invention. Alternatively, the altered antibody of the invention may have attached to it an effector or reporter molecule. For instance, the altered antibody of the invention may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent briding structure. Alternatively the procedure of recombinant DNA technology may be used to produce an altered antibody of the invention 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 altered antibody 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.

Another aspect of this invention is the discovery of a specific epitope of the F (fusion) protein of RSV which has been demonstrated to be a target for monoclonal antibodies which both protect and cure mice of infection by RSV. In addition, it has also been demonstrated that Fab fragments of such monoclonal antibodies protect mice from in vivo infection. Thus, the present

invention also relates to such specific epitope of the F protein of RSV; monoclonal antibodies directed against such epitope; and Fab fragments of such monoclonal antibodies. In addition, this invention relates to a pharmaceutical composition comprising a therapeutic, non-toxic amount of such monoclonal antibodies or Fab fragments and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or therapeutically treating RSV infection in a human or animal in need thereof which comprises administering an effective amount of such monoclonal antibodies or Fab fragments to such human or animal.

The present invention provides altered antibodies with specificity for microorganisms, and the DNA coding for such antibodies. These antibodies comprise Ig constant regions and variable regions from one source, and one or more CDRs from a different source.

In addition, amino acid substitutions in the variable region frameworks are described which are critical for antigen binding affinity. The invention also provides vectors producing the altered antibodies in mammalian cell hosts.

The present invention particularly applies to the provision of altered antibodies with the combination of properties required for the prevention and treatment of infections in animals and man. For example, non-human antibodies with specificity for micro organisms may be altered to produce "humanised" antibodies which elicit a minimal immune response in humans. In particular, the invention provides "humanised" antibodies with specificity for RSV which are shown to be effective in an animal model for RSV infection in humans and to recognise a large variety of human clinical isolates of RSV.

The present invention also provides a method for effecting minimal modifications to the amino acids of variable region frameworks in order to retain the antigen binding capacity of CDRs from a different source. The method involves stepwise alteration and testing of individual amino acids in the variable region framework potentially critical for antigen binding

affinity. The method avoids major introduction of framework amino acids from the same source as CDRs.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "humanized antibody" refers to a molecule having its complementarity determining regions (and, perhaps, minimal portions of its light and/or heavy variable domain framework region) derived from an immunoglobulin from a non-human species, the remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin.

The present invention relates to altered antibodies in which at least parts of the complementarity determining regions (CDRs) in the light and/or heavy variable domains of an acceptor monoclonal antibody have been replaced by analagous parts of CDRs from one or more donor monoclonal antibodies, and in which there may or may not have been minimal alteration of the acceptor monoclonal antibody light and/or heavy variable domain framework region in order to retain donor monoclonal antibody binding specificity, wherein such donor antibodies have specificity for microorganisms, in particular specificity for respiratory syncytial virus (RSV). The present invention also relates to a process for preparing such altered antibodies; a pharmaceutical composition comprising a therapeutic, non-toxic amount of such altered antibodies and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or therapeutically treating a microorganism-induced disease state in a human or animal in need thereof which comprises administering an effective amount of such altered antibodies to such human or animal.

The altered antibodies of the invention may be produced by the following process:

(a) producing, by conventional techniques, in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain wherein at least the CDRs (and those minimal portions of the acceptor monoclonal antibody light and/or heavy variable domain framework region

required in order to retain donor monoclonal antibody binding specificity) of the variable domain are derived from a non-human immunoglobulin, such as that produced by RSV19, and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin, thereby producing the vector of the invention;

(b) producing, by conventional techniques, in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain wherein at least theCDRs (and those minimal portions of the acceptor monoclonal antibody light and/or heavy variable domain framework region required in order to retain donor monoclonal antibody binding specificity) of the variable domain are derived from a non-human immunoglobulin, such as that produced by RSV19, and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin, thereby producing another vector of the invention;

(c) transfecting a host cell by conventional techniques with the or each vector to create the transfected host cell of the invention;

(d) culturing the transfected cell by conventional techniques to produce the altered antibody of the invention.

The host cell may be transfected with two vectors of the invention, the first vector containing an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived plypeptide. Preferably the vectors are identical except in so far as the coding sequences and selectable markers are concerned so to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector of the invention may be used, the vector including the sequence 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.

The host cell used to express the altered antibody of the invention is preferably a eukaryotic cell, most preferably a mammalian cell, such as a CHO cell or a myeloid cell.

The general methods by which the vectors of the invention may be constructed, transfection methods required to produce the host cell of the invention, and culture methods necessary to produce the altered antibody of the invention from such host cell are all conventional techniques. Likewise, once produced, the altered antibodies of the invention may be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like.

An example of the altered antibody of the invention are humanised antibodies derived from the murine monoclonal antibody RSV19 such as HuRSV19VH/VK and HuRSV19VHFNS/HuRSV19VK which are described in the Examples. Such antibodies are useful in treating, therapeutically or prophylactically, a human against human RSV infection. Therefore, this invention also relates to a method of treating, therapeutically or prophylactically, human RSV infection in a human in need thereof which comprises administering an effective, human RSV infection treating dose such altered antibodies to such human.

The altered antibodies of this invention may also be used in conjunction with other antibodies, particularly human monoclonal antibodies reactive with other markers (epitopes) responsible for the disease against which the altered antibody of the invention is directed.

The altered antibodies of this invention may also be used as separately administered compositions given in conjunction with chemotherapeutic or immunosuppressive agents. The appropriate combination of agents to utilized can readily be determined by one of skill in the art using conventional techniques. As an example of one such combination, the altered antibody of the invention known as HuRSV19VHFNS/HuRSV19VK

may be given in conjunction with the antiviral agent ribavirin in order to facilitate the treatment of RSV infection in a human.

One pharmaceutical composition of the present invention comprises the use of the antibodies of the subject invention in immunotoxins, i.e., molecules which 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., carbodiimide, glutaraldehyde, and the like, Production of various immunotoxins is well-known in the art.

A variety of cytotoxic agents are suitable for use in immunotoxins, and may include, among others, radionuclides, chemotherapeutic drugs such as methotrexate, and cytotoxic proteins such as ribosomal inhibiting proteins (e.g., ricin).

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 if desired.

The altered antibodies and pharmaceutical compositions of the 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 altered antibody of the invention or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be employed, 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 solutions 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, etc. The concentration of the the altered antibody of the invention in such pharmaceutical formulation 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., according to the particular mode of administration selected.

Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and 50 mg of an altered antibody of the invention. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of an altered antibody of the invention. Actual methods for preparing parenterally administrable compositions are well known or will be 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.

The altered antibodies of the 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.

Depending on the intended result, the pharmaceutical composition of the invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administerd to a patient already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. 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.

Single or multiple administrations of the pharmaceutical compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical composition of the invention should provide a quantity of the altered antibodies of the invention sufficient to effectively treat the patient.

It should also be noted that the altered antibodies of this invention may be used for the design and synthesis of either peptide or non-peptide compounds (mimetics) which would be useful in the same therapy as the antibody. See, e.g., Saragovi et al., Science, 253, 792-795 (1991).

Another aspect of this invention is the discovery of a specific epitope of the F (fusion) protein of RSV which has been demonstrated to be a target for monoclonal antibodies which both protect and cure mice of infection by RSV. In addition, it has also been demonstrated that Fab fragments of such monoclonal antibodies protect mice from in vivo infection. Thus, the present invention also relates to such specific epitope of the F protein of RSV; monoclonal antibodies directed against such epitope; and Fab fragments of such monoclonal antibodies. In addition, this invention relates to a pharmaceutical composition comprising a therapeutic, non-toxic amount of such monoclonal antibodies or Fab fragments and a pharmaceutically acceptable carrier or diluent; and a method of prophylactically or therapeutically treating RSV infection in a human or animal in need thereof which comprises administering an effective amount of such monoclonal antibodies or Fab fragments to such human or animal.

The present invention provides altered antibodies with specificity for microorganisms, and the DNA coding for such antibodies. These antibodies comprise Ig constant regions and variable regions from one source, and one or more CDRs from a difference source.

In addition, amino acid substitutions in the variable region frameworks are described which are critical for antigen binding affinity. The invention also provides vectors producing the altered antibodies in mammalian cell hosts.

The present invention particularly applies to the provision of altered antibodies with the combination of properties required for the prevention and treatment of infections in animals and man. For example, non-human antibodies with specificity for micro organisms may be altered to produce "humanised" antibodies which elicit a minimal immune response in humans. In particular, the invention provides "humanised" antibodies with specificity for RSV which are shown to be effective in an animal model for RSV infection in humans and to recognise a large variety of human clinical isolates of RSV.

The present invention also provides a method for effecting minimal modifications to the amino acids of variable region frameworks in order to retain the antigen binding capacity of CDRs from a different source. The method involves stepwise alteration and testing of individual amino acids in the variable region framework potentially critical for antigen binding affinity. The method avoids major introduction of framework amino acids from the same source as CDRs.

The following examples are offerred by way of illustration, not by limitation.

EXAMPLES

In the following examples all necessary restriction enzymes, plasmids, and other reagents and materials were obtained from commercial sources unless otherwise indicated.

In the following examples, unless otherwise indicated, all general cloning, ligation and other recombinant DNA methodology was performed as described in "Molecular Cloning, A Laboratory Manual (1982) eds T. Maniatis et. al., published by Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (hereinafter referred to as "Maniatis et al.").

In the following examples, the following abbreviations may be employed:

dCTP	deoxycytidine triphosphate
dATP	deoxyadenosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythysiodine triphosphate
DTT	dithiothreitol
C	cytosine
A	adenine
T	thymine
G	guanine
DMEM	Dulbecco's modified Eagle's medium
PBST	Phosphate buffered saline containing 0.02% Tween 20 (pH 7.5)

ALTERED ANTIBODIES

Examples 1-3 describe the preparation of the altered antibodies of the invention.

EXAMPLE 1-PRODUCTION OF ALTERED ANTIBODIES SPECIFIC FOR RSV

The source of the donor CDRs utilized to prepare these altered antibodies was a murine monoclonal antibody, RSV19, specific for the fusion (F) protein of RSV. The RSV19 hybridoma cell line was obtained from Dr. Geraldine Taylor, Institute for Animal Health, Compton Laboratory, Compton, Near Newbury, Berks, RG16 0NN, England. Methodology for the isolation of hybridoma cell lines secreting monoclonal antibodies specific for RSV is described by Taylor et al., Immunology, 52 (1984) p137-142.

Cytoplasmic RNA was prepared by the method of Favaloro et. al., (1980) Methods in Enzymology, Vol. 65, p.718-749, from the RSV19 hybridoma cell line, and cDNA was synthesized using Ig variable region primers as follows:

for the Ig heavy chain variable (VH) region, the primer
VH1FOR (5'TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG3')
was used, and
for the Ig light chain variable region (VK), the primer
VK1FOR (5'GTTAGATCTCCAGCTTGGTCCC3')
was used.

cDNA synthesis reactions consisted of 20mg RNA, 0.4mM VH1FOR or VK1FOR, 250mM each of dATP, dCTP, dGTP and dTTP, 50mM Tris-HCl pH 7.5, 75mM KCl, 10mM DTT, 3mM $MgCl_2$ and 27 units RNase inhibitor (Pharmacia, Milton Keynes, United Kingdom) in a total volume of 50ml. Samples were heated at 70°C for 10 minutes (min) and slowly cooled to 42°C over a period of 30 min. Then, 100m MMLV reverse transcriptase (Life Technologies, Paisley, United Kingdom) was added and incubation at 42°C continued for 1 hour.

VH and VK cDNAs were then amplified using the polymerase chain reaction (PCR) as described by Saiki, et al., Science, 239 (1988), p487-491. For such PCR, the primers used were:

VH1FOR;
VK1FOR;
VH1BACK (5'AGGTSMARCTGCAGSAGTCWGG3'); and
VK1BACK (5'GACATTCAGCTGACCCAGTCTCCA3'),

where M = C or A, S = C or G, and W = A or T .

Primers VH1FOR, VK1FOR, VH1BACK and VK1BACK, and their use for PCR-amplification of mouse Ig DNA, is described by Orlandi et al., Proc. Nat. Acad. Sci. USA, 86, 3833-3937 (1989).

For PCR amplification of VH, DNA/primer mixtures consisted of 5ml RNA/cDNA hybrid, and 0.5mM VH1FOR and VH1BACK primers. For PCR amplifications of VK, DNA/primer mixtures consisted of 5ml RNA/cDNA hybrid, and 0.5mM VH1FOR and VK1BACK primers. To these mixtures was added 200 mM each of dATP, dCTP, dGTP and dTTP, 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% (w/v) gelatin, 0.01% (v/v) Tween 20, 0.01% (v/v) Nonidet P40 and 2 units Taq DNA polymerase (United States Biochemicals-Cleveland, Ohio, USA). Samples were subjected to 25 thermal cycles of PCR at 94°C, 1 min; 60°C, 1 min; 72°C, 2 min; ending with 5 min at 72°C. For cloning and sequencing, amplified VH DNA was purified on a low melting point agarose gel and by Elutip-d column chromatography (Schleicher and Schuell-Dussel, Germany) and cloned into phage M13

(Pharmacia-Milton Keynes, United Kingdom). The general cloning and ligation methodology was as described in "Molecular Cloning, A Laboratory Manual (1982) eds T. Maniatis et. al., published by Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (hereinafter referred to as "Maniatis et al.". VH DNA was either directly ligated into the SmaI site of M13 mp18/19 (Pharmacia-Milton Keynes, UK) or, following digestion with PstI, into the PstI site of M13tg131 (Amersham International-Little Chalfont, UK). Amplified VK was similarly gel purified and cloned by the following alternatives:

PvuII digest into M13mp19 (SmaI site)
PvuII and BglII digest into M13mp18/19 (SmaI - BamHI site)
PvuII and BglII digest into M13tg131 (EcoRV - BglII site)
BglII digest into M13tg131 (SmaI - BglII site)

The resultant collections of overlapping clones were sequenced by the dideoxy method (Sanger, et al., Proc. Nat. Acad. Sci. USA. 74 (1977) p5463-5467) using Sequenase (United States Biochemicals-Cleveland, Ohio, USA).

From the sequence of RSV19 VH and VK domains, as shown in Figure 1 and 2 respectively, the CDR sequences were elucidated in accordance with the methodology of Kabat et al., in Sequences of Proteins of Immunological Interest (US Dept of Health and Human Services, US Government Printing Office, (1987)) utilizing computer assisted alignment with other VH and VK sequences.

Transfer of the murine RSV19 CDRs to human frameworks was achieved by site directed mutagenesis. The primers used were:
VHCDR1 5'CTGTCTCACCCAGTGCATATAGTAGTCGCTGAAGGTGAA GCCAGACACGGT3'
VHCDR2 5'CATTGTCACTCTGCCCTGGAACTTCGGGGCATATGGAA CATCATCATTCTCAGGATCAATCCA3'

VHCDR3 5' CCCTTGGCCCCAGTGGTCAAAGTCACTCCCCCATCTT
GCACAATA3'
VKCDR1 5' CTGCTGGTACCATTCTAAATAGGTGTTTCCATCAGTATGT
ACAAGGGTCTGACTAGATCTACAGGTGATGGTCA3'
VKCDR2 5' GCTTGGCACACCAGAAAATCGGTTGGAAACTCTGTAG
ATCAGCAG3'
VKCDR3 5' CCCTTGGCCGAACGTCCGAGGAAGATGTGAACCTTGAA
AGCAGTAGTAGGT3'

The DNA templates for mutagenesis comprised human framework regions derived from the crystallographically solved proteins, NEW (described by Saul, et al., J. Biol.. Chem., 53 (1978), p585-597) with a substitution of amino acid 27 from serine to phenylalanine (See, Riechmann et al., loc. cit.) and REI (described by Epp et al, Eur J. Biochem. 45 (1974), p513-524) for VH and VK domains, respectively. M13 based templates comprising human frameworks with irrelevant CDRs were prepared as described by Riechmann et al., Nature. 332 (1988).

Oligonucleotide site directed mutagenesis of the human VH and VK genes was based on the method of Nakamaye et al., Nucl. Acids Res. 14 (1986) p9679-9698.

To 5mg of VH or VK single-stranded DNA in M13 was added a two-fold molar excess of each of the three VH or VK phosphorylated oligonucleotides encoding the three mouse CDR (complementarity determining region) sequences. Primers were annealed to the template by heating to 70°C and slowly cooled to 37°C. To the annealed DNA was added 6u Klenow fragment (Life Technologies, Paisley, UK); 6u T4 DNA ligase (Life Technologies, Paisley, UK); 0.5mM of each of the following nucleoside triphosphates (dATP, dGTP, dTTP and 2'-deoxycytidine 5'-0-(1-thiotriphosphate) (thiodCTP); 60mM Tris-HCl (pH 8.0); 6mM $MgCl_2$; 5mM DTT (Sigma, Poole, UK); and 10mM ATP in a reaction volume of 50ml. This mixture was incubated at 16°C for 15 hours (h). The DNA was then ethanol precipitated and digested with 5 units NciI (Life Technologies, Paisley, UK) which nicks the parental strand but leaves the newly synthesised strand

containing thiodCTP intact. The parental strand was then removed by digesting for 30 min with 100 units exonuclease III (Pharmacia, Milton Keynes, United Kingdom) in 50 ml of 60mM Tris-HCl (pH 8.0), 0.66mM $MgCl_2$, and 1mM DTT. The DNA was then repaired through addition of 3 units of DNA polymerase I (Life Technologies, Paisley, UK), 2 units T4 DNA ligase in 50 ml of 60mM Tris-HCl (pH 8.0), 6mM $MgCl_2$, 5mM DTT, 10mM ATP and 0.5mM each of dATP, dCTP, dGTP and dTTP. The DNA was transformed into competent E. coli TG1 cells (Amersham International, Little Chalfont, UK) by the method of Maniatis et al. Single-stranded DNA was prepared from individual plaques and sequenced by the method of Messing (1983) Methods in Enzymology, 101, p. 20-78. If only single or double mutants were obtained, then these were subjected to further rounds of mutagenesis (utilizing the methodology described above) by using the appropriate oligonucleotides until the triple CDR mutants were obtained.

The CDR replaced VH and VK genes were cloned in expression vectors (by the method of Maniatis et al.) to yield the plasmids shown in Figures 3 and 4 respectively, and such plasmids were termed pHuRSV19VH and pHuRSV19VK. For pHuRSV19VH, the CDR replaced VH gene together with the Ig heavy chain promoter (Figures 3 and 4), appropriate splice sites and signal peptide sequences (Figures 3 and 4) were excised from M13 by digestion with HindIII and BamHI, and cloned into an expression vector containing the murine Ig heavy chain enhancer (Figures 3 and 4), the SV40 promoter (Figures 3 and 4), the gpt gene for selection in mammalian cells (Figures 3 and 4) and genes for replication and selection in E. coli (Figures 3 and 4). A human IgG1 constant region was then added as a BamHI fragment (Figures 3 and 4). The construction of the pHuRSV19VK plasmid was essentially the same except that the gpt gene was replaced by the hygromycin resistance gene (Figures 3 and 4) and a human kappa chain constant region was added (Figures 3 and 4).

10mg of pHuRSV19VH and 20mg of pHuRSV19VK were digested with PvuI utilizing conventional techniques. The DNAs were mixed

together, ethanol precipitated and dissolved in 25ml water. Approximately 10^7 YB2/0 cells (from the American Type Culture Collection, Rockville, Maryland, USA) were grown to semi-confluency, harvested by centrifugation and resuspended in 0.5ml DMEM (Gibco, Paisley, UK) together with the digested DNA in a cuvette. After 5 min on ice, the cells were given a single pulse of 170V at 960uF (Gene-Pulser, Bio-Rad-Richmond, California, USA) and left in ice for a further 20 min. The cells were then put into 20 ml DMEM plus 10% foetal calf serum and allowed to recover for 48h. After this time, the cells were distributed into a 24-well plate and selective medium applied (DMEM, 10% foetal calf serum, 0.8mg/ml mycophenolic acid, and 250mg/ml xanthine). After 3-4 days, the medium and dead cells were removed and replaced with fresh selective medium. Transfected clones were visible with the naked eye 10-12 days later.

The presence of human antibody in the medium of wells containing transfected clones was measured by conventional ELISA techniques. Micro-titre plates were coated overnight at 4°C with goat anti-human IgG (gamma chain specific) antibodies (Sera-Lab-Ltd., Crawley Down, UK) at 1 mg per well. After washing with PBST (phosphate buffered saline containing 0.02% Tween 20x (pH7.5)), 100ml of culture medium from the wells containing transfectants was added to each microtitre well for 1h at 37°C. The wells were then emptied, washed with PBST and either peroxidase-conjugated goat anti-human IgG or peroxidase-conjugated goat anti-human kappa constant region antibodies (both obatined from Sera-Lab Ltd., Crawley Down, UK) were added at 100 ng per well. Plates were then incubated at 37°C for 1h. The wells were then emptied and washed with PBST. 340 mg/ml o-phenylenediamine in 50mM sodium citrate, 50mM sodium phosphate (pH 5.0) and 0.003% (v/v) H_2O_2 were added at 200ml per well. Reactions were stopped after 1 to 5 min by the addition of 12.5% sulphuric acid at 50 ml per well. The absorbance at 492 nm was then measured spectrophotometrically.

The humanised antibody HuRSV19VH/VK, secreted from transfected cell lines cotransfected with pHuRSVVH and pHuRSVVK, was purified on Protein-A agarose columns (Boehringer Mannheim, Lewes, UK)) and tested for binding to RSV virus in an ELISA assay. Antigen consisted of calf kidney (CK) cells infected with RSV (A2 strain of RSV obtained from a child in Australia and described by Lewis et al., Med. J. Australia, 48, 932-933 (1961)) and treated with 0.5% (v/v) NP40 detergent to yield a cell lysate. A control cell lysate was similarly prepared using uninfected CK cells. Microtitre plate wells were coated with either infected or control cell lysate. Antigen coated plates were blocked with PBST for 1 hour at 37°C, washed with PBST, and thereafter humanised antibody was applied (i.e., HuRSV19VH/VK). After 1 hour at 37°C, the wells were emptied, washed with PBST and 200 ng goat anti-human IgG antibodies (Sera Lab-Ltd., Crawley Down, UK) added per well. After 1 hour at 37°C, the wells were emptied, washed with PBST and 200ml of a 1:1000 dilution of horseradish peroxidase conjugated rabbit anti-goat IgG antibodies (Sigma-Poole, UK) were added. After 1 hour at 37°C, the wells were emptied and washed with PBST. To each well was added 200ml substrate buffer (340mg/ml o-phenylenediamine in 50mM sodium citrate, 50mM sodium phosphate (pH 5.0) and 0.003% (v/v) H_2O_2). Reactions were stopped by the addition of 50ml 12.5% sulphuric acid. The absorbance at 492 nm was then measured. Antibody HuRSVVH/VK bound to RSV although with an affinity less than the murine RSV19 antibody.

EXAMPLE 2-PRODUCTION OF HIGH AFFINITY ANTIBODIES SPECIFIC FOR RSV BY A METHOD DESIGNED TO ACHIEVE MINIMAL VARIABLE REGION FRAMEWORK MODIFICATIONS GIVING RISE TO HIGH AFFINITY BINDING

The method of this invention involves the following order of steps of alteration and testing:

1. Individual framework amino acid residues which are known to be critical for interaction with CDRs are compared in the primary antibody and the altered CDR-replacement antibody. For example, heavy chain amino acid residue 94 (Kabat numbering- see Kabat et al., cited above) is compared in the primary (donor) and altered antibodies. An arginine residue at this position is thought to interact with the invariant heavy chain CDR aspartic acid residue at position 101.

If amino acid 94 comprises arginine in the framework of the primary antibody but not in the framework of the altered antibody, then an alternative heavy chain gene comprising arginine 94 in the altered antibody is produced. In the reverse situation whereby the altered antibody framework comprises an arginine residue at position 94 but the primary antibody does not, then an alternative heavy chain gene comprising the original amino acid at position 94 is produced. Prior to any further analysis, alternative plasmids produced on this basis are tested for production of high affinity altered antibodies.

2. Framework amino acids within 4 residues of the CDRs as defined according to Kabat (see Kabat et al., cited above) are compared in the primary antibody and altered CDR-replacement antibody. Where differences are present, then for each region (e.g., upstream of VHCDR1) the specific amino acids of that region are substituted for those in the corresponding region of the altered antibody to provide a small number of altered genes. Alternative plasmids produced on this basis are then tested for production of high affinity antibodies.

3. Framework residues in the primary and altered CDR-replacement antibodies are compared and residues with major differences in charge, size or hydrophobicity are highlighted. Alternative plasmids are produced on this basis with the individual

highlighted amino acids represented by the corresponding amino acids of the primary antibody and such alternative plasmids are tested for production of high affinity antibodies.

The method is exemplified by the production of a high affinity altered antibody derivative of HuRSVVH/VK (See, Example 1) specific for RSV. Comparison of VH gene sequences between RSV19VH and pHuRSV19VH (See, Figure 5) indicates that 3 out of 4 amino acid differences occur between amino acids 27 to 30 and between amino acids 91 to 94. Thus, pHuRSV19VHNIK and pHuRSV19VHFNS were produced with framework amino acids 27 to 30 and 91 to 94 in the former, and amino acids 91 and 94 in the latter, represented as in the primary RSV19VH. Using oligonucleotide site directed mutagenesis as described in Example 1, the following oligonucleotides were used for mutagenesis of the HuRSV19VH gene in M13:

pHuRSV19VHNIK - 5'ATATAGTAGTCTTTAATGTTGAAGCCAGA CA3'

pHuRSV19VHFNS - 5'CTCCCCCATGAATTACAGAAATAGA CCG3'

Humanised HuRSV19VHFNS/HuRSV19VK antibody was tested in an ELISA assay as detailed in Example 1 for analysis of binding to RSV antigen prepared from detergent-extracted, virus-infected cells. Figure 6 shows that the substitution of VH residues 91 to 94 in HuRSV19VH/VK with VH residues from mouse RSV19VH partially restored antigen binding levels. Additional analysis of HuFNS binding properties was performed using an ELISA assay in which intact Type A RS virus (Long strain) was used as the antigen. The data from such additional analysis (as shown in Figure 6A) show that there is little if any difference between the ability of the RSV19 and HuRSV19VHFNS/HuRSV19VK antibodies to bind to intact, non-denatured RS virus. This additional analysis also showed detectable binding of HuRSV19VH/VK to intact virus, although of a much lower magnitude than was seen with either

RSV19 or HuRSV19VHFNS/HuRSV19VK. Thus, the data from this additional analysis suggests that the affinity for the native antigen was restored in the HuRSV19VHFNS/HuRSV19VK mAb. Specificity of HuRSV19VHFNS/HuRSV19VK for RSV F protein was shown by conventional Western blot analysis using a truncated soluble F protein construct expressed in CHO cells.

EXAMPLE 3-SPECIFICITY AND BIOLOGICAL ACTIVITY OF AN ALTERED ANTIBODY SPECIFIC FOR RSV.

In order to ascertain the potential clinical usefulness of a humanised antibody specific for RSV, an immunofluorescence analysis of binding to 24 RSV clinical isolates was undertaken. The isolates were obtained from children during the winter of 1983-84 by the Bristol Public Health Laboratory (Bristol, England) and represented both of the major subgroups of RSV. 13 isolates were serotyped as subgroup A and 11 isolates as subgroup B. HeLa or MA104 cells infected with RSV isolates were grown in tissue culture. When the cells showed evidence of cytopathic effect, 20 ml of 0.02% (w/v) disodium EDTA (ethylenediaminetetra-acetic acid) (BDH Chemicals Ltd., Poole, UK) in PBS and 3ml of 0.25% (w/v) trypsin in PBS were added and the cell suspension spotted into wells of PTFE-coated slides (polytetrafluoroethylene coated slides) (Hendley, Essex, UK). After 3 hours at 37°C, the slides were dried and fixed in 80% acetone. Cells were overlaid with monoclonal antibody (i.e., either humanised antibody, HuRSV19VHFNS/HuRSV19VK, or the murine antibody RSV19) for 1 hour at room temperature. After extensive washing, either fluorescein-conjugated rabbit anti-mouse IgG (Nordic Laboratories-Tilburg, The Netherlands) or fluorescein-conjugated goat anti-human IgG1 (Southern Biotechnology, Birmingham, Alabama, USA) was added, and the incubation was repeated. After further washing, cells were mounted in glycerol and examined under UV light.

Table I shows the results of comparative immunofluorescence for the humanised antibody, HuRSV19VHFNS/HuRSV19VK, and the murine antibody RSV19. This data indicates that 100% of clinical isolates are recognised by both the humanised and murine antibodies. Such data demonstrates that the humanised antibody has the potential for recognition of most clinical isolates comprising both of the major RSV subgroups.

TABLE I

Binding of Humanised Anti-RSV to Clinical Isolates

Isolate Number	Extent of Fluorescence* HuRSV19VHFNS/HuRSV19VK	Murine RSV19
Subgroup A		
V818	++++	++++
V795	++++	++++
V00401	++	+++
V00214	+	++
V00764	++	+++
V743	++	+++
V316	++	++
V369	++++	++++
V1249	+++	+++
V04692	+++	+++
V1248	+	+
V01232	++	++
V729	+	++
Subgroup B		
V00634	+	++
V4715	++	+++
V00463	+	++
V4712	++	++
V00165	++	++
V00422	++	++
V837	+++	+++
V00900	++	++
4677	+++	+++
4424	++	++
V01231	+	+

* +,++,+++ and ++++ refer to relative numbers of fluorescing cells observed and represent the proportion of cells infected

The humanised antibody, HuRSV19VHFNS/HuRSV19VK, was next tested for biological activity in vitro in a fusion inhibition assay. A suspension of MA104 cells was infected with RSV at an m.o.i. (multiplicity of infection) of 0.01 PFU (plaque forming units) per cell. After 1 hour at 37°C, 2ml of cells at 10^5/ml were distributed to glass coverslips in tubes. After a further 24 hours at 37°C, the culture medium was replaced by medium containing dilutions of humanised antibody, HuRSV19VHFNS/HuRSV19VK. 24 hours later, coverslip cultures were fixed in methanol for 10 minutes and stained with May Grunwald stain (BDH Chemicals Ltd., Poole, UK). Table II shows the effect of increasing concentrations of HuRSV19VHFNS/HuRSV19VK in inhibiting the frequency of giant cells. The data represented in the following Table II demonstrates the biological activity of the humanised antibody HuRSV19VHFNS/HuRSV19VK in inhibiting Type A RSV induced cell fusion. It should be noted that additional studies showed that the fusion inhibition titres for RSV19 versus HuRSV19VHFNS/HuRSV19VK were comparable, providing additional evidence that affinity for the native viral antigen was fully restored in HuRSV19VHFNS/HuRSV19VK. The humanized antibody HuRSV19VHFNS/HuRSV19VK has also been shown, (using methodology analogous to that utilized above for showing inhibition of Type A RSV induced cell fusion), to exhibit a dose dependent inhibition of Type B RSV (strain 8/60) induced giant cell fusion.

TABLE II

Inhibition of RSV Induced Cell Fusion by Humanised Anti-RSV

Concentration of HuRSV19VHFNS/HuRSV19VK (ug/ml)	Number of Giant Cells*	Average number of Nucleii
100	44	4.5
50	71	4.0
25	40	3.8
12.5	67	
6.3	89	
3.1	87	
1.6	164	
0.8	201	
0.4	292	
0.2	219	
0	239,259	14,13.5
0 (no virus)	10	

* Scored as the number of cells with 2 or more nucleii in 20 fields with a 25x objective microscope lens

The humanised antibody, HuRSV19VHFNS/HuRSV19VK was next tested for biological activity in vivo in an RSV-mouse infection model. BALB/c mice (obtained from Charles Rivers: specific pathogen free category 4 standard) were challenged intranasally with 10^4 PFU of the A2 strain of human RSV (as described by Taylor et al., Infection and Immunity, 43 (1984) p649-655). Groups of mice were administered with 25mg of humanised antibody either one day prior to virus infection or 4 days following infection.

Administration of antibody was either by the intranasal (i.n.) or intraperitoneal (i.p.) routes. 5 days after RSV infection, mice were sacrificed and lungs were assayed for RSV PFU (see, Taylor et al., Infection and Immunity, 43 (1984) p649-655). The data in the following Table III shows that HuRSV19VHFNS/HuRSV19VK at a single dose of 25mg per mouse is extremely effective in prevention and treatment of RSV infection.

TABLE III

Prevention and Treatment of RSV Infection in Mice by Humanised Anti-RSV

Antibody Treatment		
Day*	Route+	log_{10} PFU per gram of lung-
-1	i.p.	<1.7
		<1.7
		<1.7
		<1.7
		<1.7
-1	i.n.	<1.7
		<1.7
		<1.7
		<1.7
		<1.7
+4	i.p.	<1.7
		<1.7
		1.7
		<1.7
+4	i.n.	<1.7
		1.7
		<1.7
		1.7
		<1.7
No antibody		4.47
		4.32
		4.64
		4.61
		4.55

* -1 refers to administration of HuRSV19VHFNS/HuRSV19VK antibody 1 day prior to RSV infection, +4 refers to administration of antibody 4 days post infection

\+ i.p. - intraperitoneal, i.n. - intranasal

\- virus PFU is expressed as the virus titre from dilutions of 10%, (w/v) lung homogenates (see Taylor et al., loc. cit.) adjusted to PFU per gram of lung. <1.7 log_{10} PFU per gram means that no virus was detected in the starting dilution of lung homogenate 10%.

HuRSV19VHFNS/HuRSV19VK was also shown to be active in vivo when administered prophylactically to mice challenged with Type B RSV (strain 8/60) using methodology similar to that described above. In addition, the humanized antibody HuRSV19VH/VK was also shown to be active in vivo when administered prophylactically to mice challenged with Type B RSV (strain 8/60) using methodology similar to that described above.

This invention also relates to a method of preventing human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection inhibiting dose of an altered antibody of this invention for which RSV19 or RSV20 was the donor monoclonal antibody.

This invention also relates to a method of therapeutically treating human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection therapeutic dose of an altered antibody of this invention for which RSV19 or RSV20 was the donor monoclonal antibody.

To effectively prevent RSV infection in a human, one dose of approximately 1 mg/kg to approximately 20 mg/kg of an altered antibody of this invention for which RSV19 or RSV20 was the donor monoclonal antibody, such as HuRSV19VH/VK or HuRSV19VHFNS/HuRSV19VK should be administered parenterally, preferably i.v. (intravenously) or i.m. (intramuscularly); or one dose of approximately 200 ug/kg to approximately 2 mg/kg of such antibody should be administered i.n. (intranasally). Preferably, such dose should be repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April). Alternatively, at the beginning of the RSV season, one dose of approximately 5 mg/kg to approximately 100 mg/kg of an altered antibody of this invention for which RSV19 or RSV20 was the donor monoclonal antibody, such as HuRSV19VH/VK or

HuRSV19VHFNS/HuRSV19VK, should be administered i.v. or i.m. or one dose of approximately 0.5 mg/kg to approximately 10 mg/kg of such antibody should be administered i.n.

To effectively therapeutically treat RSV infection in a human, one dose of approximately 2 mg/kg to approximately 20 mg/kg of an altered antibody of this invention for which RSV19 or RSV20 was the donor monoclonal antibody, such as HuRSV19VH/VK or HuRSV19VHFNS/HuRSV19VK should be administered parenterally., preferably i.v. or i.m.; or approximately 200 ug/kg to approximately 2 mg/kg of such antibody should be administered i.n. Such dose may, if necessary, be repeated at appropriate time intervals until the RSV infection has been eradicated.

The altered antibodies of the invention may also be administered by inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as an aerosol formulation or a metered dose inhaler, may be prepared by conventional techniques. For example, to prepare a composition for administration by inhalation, for an aerosol container with a capacity of 15-20 ml: Mix 10 mg of an altered antibody of this invention with 0.2-0.2% of a lubricating agent, such as polysorbate 85 or oleic acid, and disperse such mixture in a propellant, such as freon, preferably in a combination of (1,2 dichlorotetrafluoroethane) and difluorochloromethane and put into an appropriate aerosol container adaped for either intranasal or oral inhalation administration. As a further example, for a composition for administration by inhalation, for an aerosol container with a capacity of 15-20 ml: Dissolve 10 mg of an altered antibody of this invention in ethanol (6-8 ml), add 0.1-0.2% of a lubricating agent, such as polysorbate 85 or oleic acid; and disperse such in a propellant, such as freon, preferably a combination of (1.2 dichlorotetrafluoroethane) and difluorochloromethane, and put into an appropriate aerosol container adapted for either intranasal or oral inhalation administration.

The preferred daily dosage amount to be employed of an altered antibody of the invention to prophylactically or therapeutically treat RSV infection in a human in need thereof to be administered by inhalation is from about 0.1 mg to about 10 mg/kg per day).

Natural RSV infections have been reported in cattle, goats, sheep and chimpanzees. Thus, for example, utilizing the methodology described above, an appropriate mouse antibody could be "bovinized", and appropriate framework region residue alterations could be effected, if necessary, to restore specific binding affinity. Once the appropriate mouse antibody has been created, one of skill in the art, using conventional dosage determination techniques, can readily determine the appropriate dose levels and regimens required to effectively treat, prophylactically or therapeutically, bovine RSV infection.

Examples 1-3 show that altered antibodies for prevention and treatment of infection can be produced with variable region frameworks potentially recognised as "self" by recipients of the altered antibody. Minor modifications to the variable region frameworks can be implemented to effect large increases in antigen binding without appreciable increased immunogenicity for the recipient. Such altered antibodies can effectively prevent and eradicate infection.

Thus the present invention provides an altered antibody in which complementarity determining regions (CDRs) in the heavy or light chain variable domains have been replaced by analogous parts of CDRs from a different source resulting in antibodies possessing the combination of properties required for effective prevention and treatment of infectious disease in animals or man. Suitably, the entire CDRs have been replaced. Preferably, the variable domains in both heavy and light chains have been altered by CDR replacement. Typically, the CDRs from a mouse antibody are grafted onto the framework regions of a human antibody. The

altered antibody preferably has the structure of a natural antibody or a fragment thereof.

A preferred antibody is one directed against respiratory syncytial virus (RSV), preferably one specific for the fusion (F) protein of RSV. A particularly preferred antibody of this kind has the following N-terminal variable domain amino acid sequences (see the Amino Acid Shorthand Table immediately following) in its heavy and light chains:

heavy:

QVQLQESGPGLVRPSQTLSLTCTVSGFT
FS(or NIK)DYYMHWVRQPPGRGLEWIGWIDPEN
DDVQYAPKFQGRVTMLVDTSKNQFSLRLSSVTAAD
TAVYCAR(or FCNS)WGSDFDHWGQGTTVTVSS

light:

DIQLTQSPSSLSASVGDRVTITCRSSQTLVHTDGNTY
LEWYQQKPGAPKLLIYRVSNRFSGVPSRFSGSGSGT
DFTFTISSLQPEDIATYYCQSHLPRTFGQGTKVEIK

Table Amino Acid Shorthand

Amino Acid	Three-letter abbreviation	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E

Glutamic acid	Glu		E
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	He	I	
Leucine	Leu	L	
Lysine	Lys		K
Methionine	Met	M	
Phenylalanine	Phe		F
Proline	Pro		P
Serine	Ser		S
Threonine	Thr	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val		V

It will be understood by those skilled in the art that such an altered antibody may be further altered by changes in variable domain amino acids without necessarily affecting the specificity of the antibody for the fusion (F) protein of RSV, and it is anticipated that even as many as 25% of heavy and light chain amino acids may be substituted by other amino acids either in the variable domain frameworks or CDRs or both. Such altered antibodies can be effective in prevention and treatment of respiratory syncytial virus (RSV) infection in animals and man.

The invention also includes a recombinant plasmid containing the coding sequence of the altered antibody of the invention, and a

mammalian cell-line transfected with a recombinant plasmid containing the coding sequence of the altered antibodies hereof. Such a vector is prepared by conventional techniques and suitably comprises DNA sequences encoding immunoglobulin domains including variable region frameworks and CDRs derived from a different source and a suitable promoter operationally linked to the DNA sequences which encode the altered antibody. Such a vector is transfected into a transfected mammalian cell via conventional techniques.

The invention further comprises a method for effecting minimal modifications within the variable region frameworks of an altered antibody necessary to produce an altered antibody with increased binding affinity comprising the following steps:

(a) analysis of framework amino acids known to be critical for interaction with CDRs, and production and testing of altered antibodies where single framework amino acids have been substituted by the corresponding amino acids from the same source as the CDRs;

(b) analysis of framework amino acids adjacent to CDRs, and production and testing of altered antibodies where one or more of the amino acids within 4 residues of CDRs have been substituted by the corresponding amino acids from the same source of the CDRs;

(c) analysis of framework residues within the altered antibody, and production and testing of altered antibodies where single amino acids have been substituted by the corresponding amino acids with major differences in charge, size or hydrophobicity from the same source of CDRs.

The following Examples relate to the novel RSV F protein epitope of the invention.

SPECIFIC RSV F PROTEIN EPITOPE

The following examples demonstrate that two monoclonals which protect and cure mice of *in vivo* infection by RSV recognize a linear epitope within the F protein of RSV (which linear epitope may be part of a conformational epitope) and which contains amino acid residues 417 to 438 of the F protein coding sequence including an essential arginine residue at position 429, or any immunoprotective portion thereof, such as, but not limited to amino acid residues 417-432 of the F protein coding sequence, and amino acid residues 422-438 of the F protein coding sequence. This novel epitope (which may be referred to herein as "epitope 417-438") is a suitable target for screening for other neutralizing epitopes, for protective and therapeutic agents against RSV, and in particular, for monoclonal antibodies against this epitope. Knowledge of this epitope enables one of skill in the art to define synthetic peptides which would be suitable as vaccines against RSV. Epitope 417-438 is also useful for generating monoclonal antibodies which will be useful in the treatment, therapeutic and/or prophylactic, of human RSV infection in humans.

The present invention also applies to the use of Fab fragments derived from monoclonal antibodies directed against such novel epitope as protective and therapeutic agents against *in vivo* infection by viruses, and particularly relates to the protection against RSV.

The invention also includes a recombinant plasmid containing the coding sequence of a monoclonal antibody generated against the 417-438 epitope, and a mammalian cell-line transfected with a recombinant plasmid containing such coding sequence. Such a vector is prepared by conventional techniques and suitably comprises DNA sequences encoding immunoglobulin domains including variable region frameworks and CDRs and a suitable promoter operationally linked to the DNA sequences which encode

the antibody. Such a vector is transfected into a mammalian cell via conventional techniques.

EXAMPLE 4

This example shows the production of murine monoclonal antibodies against the F protein of RSV which protect and cure mice of infection.

Murine monoclonal antibodies (mAbs) 19 and 20 were produced as follows. BALB/c mice (obtained from Charles Rivers-specific pathogen free) were inoculated intranasally (i.n.) on two occasions, 3 weeks apart, with $1x10^4$ PFU of the A2 strain of human (H) RSV (described by Lewis et al., 1961, Med. J. Australia, 48, 932-933). After an interval of 4 months, the mice were inoculated intraperitoneally (i.p.) with $2x10^7$ PFU of the 127 strain of bovine (B) RSV (isolated at Institute for Animal Health, Compton, Near Newbury, Berks, England). Three days after inoculation, the immune splenocytes were fused with NS-1 myeloma cells (see, Williams et al., 1977, Cell, 12, 663). The resulting hybridomas were screened for antibody to RSV by radioimmunoassay and immunofluorescence as described previously (Taylor et. al., 1984, Immunology, 52, 137-142), cloned twice on soft agar (as described by Kohler et. al., "Immunologic Methods", pp397-402, ed. I. Lefkovitz & B. Perris, Academic Press), and the resulting cloned cells were inoculated into BALB/c mice to produce ascitic fluid as described previously (see, Taylor et al., 1984, Immunology, 52, 137-142).

The specificity of the mAbs for viral polypeptides was determined by radioimmune precipitation of (^{35}S)-methionine or (^{3}H)-glucosamine labelled RSV-infected cell lysates as described previously (see, Kennedy, et al., 1988, J. Gen Virol, 69, 3023-2032) and by immunoblotting (see, Taketa et al., 1985, Electrophoresis, 6, 492-497). The antigens used in immunoblotting were either Hep-2

cells (obtained from the American Type Culture Collection, Rockville, Maryland, USA) infected with the A2 strain of HRSV or primary calf kidney (CK) cells (produced at the Institute for Animal Health, Compton) infected with the 127 strain of BRSV. Uninfected Hep-2 or CK cells were used as control antigens.

The immunoglobulin isotype of the mAbs was determined by immunodiffusion using a radial immunodiffusion kit (Serotec, Kidlington, Oxfordshire, UK).

The properties of mAbs 19 and 20 are shown in the following Table A.

TABLE A

Table A Properties of mAb 19 and mAb 20

mAb	Protein specificity			Ig class	ELISA titre[1]			% SFA[2]	Complement lysis[3]	Neut. titre[4]	Fusion inhibition	Mouse protection[5]
	RIPA	Western blot			A2	8/60	BRS					
		Native	Reduced									
19	F	140K, 70K	46K	G2a	7.2	7.4	6.7	88	1.7	3.4	+	>3.8
20	F	140K, 70K	46K	G2a	>6.0	8.6	7.5	69	76	4.3	+	>3.8

[1]Antibody titre, using HRSV strain A2 (subtype A), HRSV strain 8/60 (subtype B) and BRSV strain 127 as antigens in ELISA, expressed as $\log_{10}$ titre.

[2]SFA = percent of HRSV strain A2 infected cells showing surface fluorescence.

[3]Percent specific chromium release from virus infected cells (bovine nasal mucosa cells persistently infected with BRSV) by 1/100 dilution of mAb and rabbit complement.

[4]50% plaque reduction titre expressed as $\log_{10}$.

[5]Reduction in peak titre of RSV, strain A2 in lungs of mice given 100 µl of mAb intra peritoneally one day before intranasal challenge, expressed as $\log_{10}$ pfu.

Immune precipitation of radiolabelled RSV (by the method of Brunda et al, (1977) J. Immunol. 119, 193-198) indicated that mAbs 19 and 20 recognized the fusion (F) glycoprotein. This was confirmed by a Western blot of non-reduced and reduced lysates of cells infected with RSV. The blots were probed with HRP-conjugated goat anti-mouse IgG (Kpl, Gaithersburg, Maryland, USA). mAbs 19 and 20 recognized the 140k F protein dimer and the 70K monomer present in the native F protein antigen and the 46K F1 fragment in antigen denatured by boiling in 2-mercaptoethanol. Both mAb 19 and 20 were identified as IgG2a, and their ELISA titres against the A2 and 8/60 strains of HRSV were similar to the ELISA titres against the 127 strain of BRSV, indicating that the epitopes recognized by these mAbs were conserved amongst strains of human and bovine RSV. Both mAB 19 and 20 neutralized RSV infectivity and inhibited the formation of multinucleated giant cells in MA104 cells infected with RSV. In contrast to mAb 19, mAb 20 lysed RSV-infected cells in the presence of rabbit complement. The failure of mAb 19 to lyse RSV-infected cells was not due to failure to bind to the surface of virus-infected cells since mAb 19 stained 88% of such cells. The failure of mAb19 and complement to lyse virus-infected cells indicates that antibody and complement-mediated lysis is not important in the in vivo protection mediated by this antibody. The ability of mAbs 19 and 20 to protect against RSV infection was assessed by challenging mice i.n. with approximately 10^4 PFU of RSV 24 h after i.p. inoculation of mAbs 19 and 20. The lungs of untreated mice killed 5 days after challenge contained 5.5 $\log_{10}$PFU of RSV/g tissue whereas virus was not detected in the lungs of mice given either mAb 19 or 20.

EXAMPLE 5

This example describes methods of isolating mutants of RSV which are resistant to inhibition by mAbS 19 and 20 generated in Example 4.

Mutant RS viruses refractory to neutralization by mAbs 19 and 20 were produced using a plaque reduction technique with the A2 strain of HRSV as follows. Confluent monolayers of CK cells, in a tissue culture flask, were infected with the A2 strain of HRSV at a MOI of 0.1. Starting 24 hours after infection and continuing for 3 to 5 days, the culture medium was replaced daily with fresh medium containing 10% mAb. Virus was harvested when a cytopathic effect was observed. Virus prepared in this way was mixed with an equal volume of either undiluted mAb 19 or 20, or medium alone for 1 hour at room temperature and inoculated onto CK monolayers in 35mm multi-well plates (Nunc, Kamstrup, Riskilde, Denmark). After 1 hour incubation at 37°C, the plates were overlaid with medium containing 0.25% agarose and 10% mAb or medium alone. Cultures were incubated at 37°C in 5% CO_2 in air for 7 days before adding the vital stain, 0.3% 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide in 0.15M NaCl, to the overlay to visualize virus plaques.

Putative mutant viruses were removed in agar plugs from plates which contained single plaques, diluted in medium, mixed with an equal volume of mAbs 19 or 20 and inoculated onto CK monolayers in 35 mm multi-well plates as before. Putative mutant viruses were plaque picked again and inoculated into tubes containing coverslips of calf testes cells. After 4 to 6 days incubation, the coverslips were removed and stained with mAb 19 and 20 and FITC-labelled rabbit anti-mouse Ig (Nordic Labs, Tilburg, The Netherlands). As a positive control, coverslips were stained with polyclonal bovine antiserum to RSV (produced at Institute for Animal Health-Compton form a gnotobiotic calf hyperimmunised with RSV), and FITC-labelled rabbitt anit-bovine Ig (obtained from Nordic Immunology, Tilburg, The Netherlands). RS viruses that failed to react by immunofluorescence to mAb 19 or 20 were classed as mutant viruses and were used to infect monolayers of Hep-2 cells to produce antigen for ELISA. Thus, 3 to 4 days after RSV infection,

cells were scraped into the medium, spun at 400 g for 5 mins, resuspended in distilled water, and treated with 0.5% (v/v) NP40 detergent to yield a cell lysate. A control cell lysate was made in a similar way using uninfected Hep-2 cells. The binding of a panel of mAbs to the F protein of RSV to the mutant viruses was examined by ELISA. Microtitre plate wells were coated with 50 ul of either infected or control cell lysate overnight at 37°C, incubated with blocking buffer consisting of 5% normal pig serum in PBS and 0.05% Tween 20 for 1 h at room temperature and washed 5x with PBS/TWEEN. Serial dilutions (three times) of the mAbs were added to the wells and the plates were incubated for 1 hour. After washing 5 times with PBS/Tween, HRP-conjugated goat anti-mouse IgG (Kpl, Gaithersburg, Maryland, USA), diluted 1:2000, was added to each well. After a final washing, bound conjugate was detected using the substrate 3,3',5,5'-tetramethylbenzidine (TMB), (obtained from ICN Immunobiologicals, Illinois). Mutant viruses, selected for resistance to mAb 19, failed to react in ELISA with both mAbs 19 and 20. Similarly, mutant viruses selected for resistance to mAb 20 failed to react with mAbs 19 and 20. All other mAbs tested reacted with the mutants to the same extent as to parent HRSV, strain A2. These results are illustrated in the following Table B.

TABLE B

Table B Binding of anti-F mAbs to antibody escape mutants of RSV.

mAb	Parent A2	Mutants selected with indicated mAb					
		19			20		
		C4848f	C4909/1	C4902/6	C4902Wa	C42902Wb	C4902Wc
1	+	+	+	+	+	+	+
2	+	+	+	+	±	±	±
5	+	+	+	±	±	±	±
11	+	+	+	±	±	±	+
13	±	+	+	±	±	±	±
14	+	±	+	+	±	±	±
16	+	±	+	±	±	±	±
17	+	+	+	±	±	±	±
18	+	+	+	+	±	±	+
19	+	-	-	-	-	-	-
20	±	-	-	-	-	-	-
21	+	+	+	+	±	±	±
B1	+	+	+	+	+	+	+
B2	+	+	+	+	+	±	+
B3	+	+	+	+	+	±	+
B4	+	+	+	±	+	+	+
B5	±	+	+	±	±	+	+
B6	+	+	+	+	±	±	+
B7	+	+	+	+	+	+	+
B8	+	+	+	+	+	+	+
B9	+	+	+	+	+	+	+
B10	+	+	+	+	+	+	+
7C2	+	+	+	+	+	+	+
47F	+	+	+	+	+	+	+

EXAMPLE 6

This example describes the identification of an amino acid sequence within the F protein which binds protective monoclonal antibodies and demonstrates that arginine 429 is essential for binding protective mabs to this amino acid sequence.

Poly(A)$^+$ RNAs, isolated from cells infected with either the A2 strain of HRSV or each of the mutants described in Example 5, were used to sequence the F protein mRNA. These sequences were determined by the dideoxy method (cited above) using 5'-^{32}P-labelled oligonucleotide primers, synthesized according to the previously reported F-protein sequence of the Long strain of RSV (see, Lopez, et al., 1988, Virus Res. 10, 249-262), followed by a chase with terminal deoxynucleotide transferase (see, DeBorde, et al., 1986, Anal Biochem. 157, 275-282). Three mutants were selected with mAb 19 and three were selected with mAb 20. All such mutants showed a single transversion (C to G) at nucleotide 1298 compared with the parent A_2 strain. This nucleotide substitution changes the amino acid residue at position 429 of the F protein from arginine to serine. Since mAbs 19 reacted in Western blot with the F_1 subunit, it is likely that the antibody-binding site is determined by a linear sequence of contiguous amino acids in which residue 429 of the F_1 subunit plays an essential role.

Synthetic peptides corresponding to amino acids residues 417-432, 422-438, 417-438 and 421-450 of the F protein were examined for their ability to react with mAbs 19 and 20 in ELISA. mAbs 19 and 20 reacted with peptides 417-432 (F417), 417-438 and with 422-438 (F422) but not with peptide 431-450. The binding of mAb 19 to peptides 417-432 and 422-438 (2ug/well) either coated onto microtitre plate wells overnight at 37°C ("dry") or coated onto the wells for 1h at room temperature ("wet") is shown in Figure 7. It should be noted that mAb 20 gave essentially the same results.

Example 7

This example shows that Fab fragments derived from mAbs 19 and 20 can protect and treat mice infected by RSV.

mAbs 19 and 20 were purified from ascitic fluid using Protein A Sepharose (Pharmacia, Milton Keynes, United Kingdom). Approximately 10 mg of purified mAb 19 and 20 were incubated with 0.5 ml of immobilized papain (Pierce-Oud-Beijerland, The Netherlands) for 5 h and overnight respectively at 37°C with constant mixing. The resulting Fab fragments were recovered on an immobilized Protein A column (Pierce). The purified IgG and the papain cleaved fragments were analyzed by SDS-PAGE under reducing conditions. The purified IgG showed bands at 53,000d and 23,000d, corresponding to Ig heavy and light chains. The Protein A fractions containing Fab fragments showed bands at approximately 25,000d and the fraction containing the Fc fragments showed 3 distinct bands corresponding to the heavy and light chains of the undigested IgG and also the Fc fragment at approximately 28,000d. The purified IgG and the papain cleaved fragments were evaluated for anti-RSV activity by ELISA with HRSV strain A2 infected and uninfected Hep-2 cells as antigen, and HRP-goat anti-mouse Fab (Sigma Chemical Co., St. Louis, Mi, USA) and HRP-goat anti-mouse Fc (ICN ImmunoBiologicals, Illinois). The ELISA showed that the Fab fragments of mAbs 19 and 20 were not contaminated with undigested Ig. These data are illustrated in the following Table C.

TABLE C

Table C Prophylactic and therapeutic effects of Fab fragments on RSV infection in mice.

Antibody	ELISA titre (log_{10})		D5 RSV titre in lungs	
	Anti-Fc	Anti-Fab	mAb d-1	mAb d4
19	4.4	4.3	< 1.7 (0/5)	< 1.7 (0/4)
19 Fab	< 2.0	4.6	< 1.7 (0/5)	< 1.7 (0/5)
None			4.6 ± 0.06	
20	5.1	5.1	< 1.7 (0/5)	< 1.7 (1/5)
20 Fab	< 2.0	4.8	< 1.7 (2/5)	< 1/7 (2/5)
None			4.5 ± 0.08	

Antibody titre measured by ELISA using RSV/A2 and antigen

The concentration of antibody in undigested mAbs 19 and 20 were adjusted to give ELISA titres similar to those of the Fab fragments and examined for their ability to protect against RSV infection in BALB/c mice. Groups of 5 mice were inoculated i.n. with undigested, purified mAb 19 or mAb 20 or Fab fragments (from mAb 19 or mAb 20) either 1 day before or 4 days after i.n. inoculation with approximately 10^4 PFU of the A2 strain of HRSV. Control mice were inoculated with HRSV only. Five days after virus challenge, mice were killed and the lungs assayed for RSV PFU on secondary CK cells as described previously (see, Taylor et al., 1984, Infect Immun. 43, 649-655). Fab fragments of mAbs 19 and 20 were highly effective both in preventing RSV infection and in clearing an established infection.

This invention relates to the 417-438 epitope. This invention also relates to monoclonal antibodies generated against the 417-438 epitope. Such monoclonal antibodies are produced by conventional techniques and include, without limitation, murine monoclonal antibodies, human monoclonal antibodies, and bovine monoclonal antibodies. Such monoclonal antibodies may comprise a complete antibody molecule (having full length heavy and light chains) or any fragment thereof, such as the Fab or $(Fab')_2$ fragment, a light chain or heavy chain dimer, or any minimal recombinant fragment thereof such as an Fv or a SCA (single-chian antibody) or any other molecule with the same specificity as the monoclonal antibody.

This invention also relates to a pharmaceutical composition comprising a monoclonal antibody generated against the 417-438 epitope and a pharmaceutically acceptable carrier or diluent.

This invention also relates to a method of preventing human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection inhibiting dose of a monoclonal antibody generated against the 417-438 epitope.

This invention also relates to a method of therapeutically treating human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection therapeutic dose of a monoclonal antibody generated against the 417-438 epitope.

To effectively prevent RSV infection in a human, one dose of approximately 1 mg/kg to approximately 20 mg/kg of a monoclonal antibody generated against the 417-438 epitope should be administered parenterally, preferably i.v. (intravenously) or i.m. (intramuscularly); or one dose of approximately 200 ug/kg to approximately 2 mg/kg of such antibody should be administered i.n. (intranasally). Preferably, such dose should be repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April). Alternatively, at the beginning of the RSV season, one dose of approximately 5 mg/kg to approximately 100 mg/kg of a monoclonal antibody generated against the 417-438 epitope should be administered i.v. or i.m. or one dose of approximately 0.5 mg/kg to approximately 10 mg/kg of such antibody should be administered i.n.

To effectively therapeutically treat RSV infection in a human, one dose of approximately 2 mg/kg to approximately 20 mg/kg of a monoclonal antibody generated against the 417-438 epitope should be administered parenterally., preferably i.v. or i.m.; or approximately 200 ug/kg to approximately 2 mg/kg of such antibody should be administered i.n. Such dose may, if necessary, be repeated at appropriate time intervals until the RSV infection has been eradicated.

A monoclonal antibody generated against the 417-438 epitope may also be administered by inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage

forms for such administration, such as an aerosol formulation or a metered dose inhaler, may be prepared by conventional techniques. For example, to prepare a composition for administration by inhalation, for an aerosol container with a capacity of 15-20 ml: Mix 10 mg of a monoclonal antibody generated against the 417-438 epitope with 0.2-0.2% of a lubricating agent, such as polysorbate 85 or oleic acid, and disperse such mixture in a propellant, such as freon, preferably in a combination of (1,2 dichlorotetrafluoroethane) and difluorochloromethane and put into an appropriate aerosol container adaped for either intranasal or oral inhalation administration. As a further example, for a composition for administration by inhalation, for an aerosol container with a capacity of 15-20 ml: Dissolve 10 mg of a monoclonal antibody generated against the 417-438 epitope in ethanol (6-8 ml), add 0.1-0.2% of a lubricating agent, such as polysorbate 85 or oleic acid; and disperse such in a propellant, such as freon, preferably a combination of (1.2 dichlorotetrafluoroethane) and difluorochloromethane, and put into an appropriate aerosol container adapted for either intranasal or oral inhalation administration.

The preferred daily dosage amount to be employed of a monoclonal antibody generated against the 417-438 epitope to prophylactically or therapeutically treat RSV infection in a human in need thereof to be administered by inhalation is from about 0.1 mg to about 10 mg/kg per day.

CLAIMS

What is claimed is:

1. An altered antibody in which at least parts of the complementarity determining regions (CDRs) in the light and/or heavy variable domains of an acceptor monoclonal antibody have been replaced by analagous parts of CDRs from one or more donor monoclonal antibodies, and in which there may or may not have been minimal alteration of the acceptor monoclonal antibody light and/or heavy variable domain framework region in order to retain donor monoclonal antibody binding specificity, wherein such donor antibodies have specificity for a particular microorganism.

2. The antibody of Claim 1 wherein the microorganism is human respiratory syncytial virus (RSV).

3. The antibody of Claim 2 wherein the donor antibody is directed against the fusion (F) protein of RSV.

4. The antibody of Claim 2 wherein the donor antibody is directed against epitope 417-438.

5. The antibody of Claim 2 which has the following N-terminal variable domain amino acid sequences in its heavy and light chains:

heavy:

QVQLQESGPGLVRPSQTLSLTCTVSGFT
FS(or NIK)DYYMHWVRQPPGRGLEWIGWIDPEN
DDVQYAPKFQGRVTMLVDTSKNQFSLRLSSVTAAD
TAVYYCAR(or FCNS)WGSDFDHWGQGTTVTVSS

light

QLTQSPSSLSASVGDRVTITCRSSQTLVHTDGNTYL
EWYQQKPGAPKLLIYRVSNRFSGVPSRFSGSGSGTD
FTFTISSLQPEDIATYYCQSHLPRTFGQGTKVEIK

6. The antibody of Claim 2 wherein the donor monoclonal antibody is RSV19.

7. The antibody of Claim 2 wherein the donor monoclonal antibody is RSV20.

8. The antibody of Claim 6 which is HuRSV19VH/VK.

9. The antibody of Claim 6 which is HuRSV19VHFNS/HuRSV19VK.

10. The antibody of Claim 1 which is a Fab fragment or a $(Fab')_2$ fragment.

11. A pharmaceutical composition comprising the altered antibody of Claim 1 and a pharmaceutically acceptable carrier or diluent.

12. The composition of Claim 11 wherein the microorganism is human RSV.

13. The composition of Claim 11 wherein the donor antibody is directed against the fusion (F) protein of RSV.

14. The composition of Claim 11 wherein the donor antibody is directed against epitope 417-438.

15. The composition of Claim 11 wherein the altered antibody has the following N-terminal variable domain amino acid sequences in its heavy and light chains:

heavy:

QVQLQESGPGLVRPSQTLSLTCTVSGFT
FS(or NIK)DYYMHWVRQPPGRGLEWIGWIDPENDD
VQYAPKFQGRVTMLVDTSKNQFSLRLSSVTAADTA
VYYCAR(or FCNS)WGSDFDHWGQGTTV TVSS

light:

IQLTQSPSSLSASVGDRVTITCRSSQTLVHTDGNTYL
EWYQQKPGAPKLLIYRVSNRFSGVPSRFSGSGSGTD
FTFTISSLQPEDIATYYCQSHLPRTFGQGTKVEIK

16. The composition of Claim 11 wherein the donor monoclonal antibody is RSV19.

17. The composition of Claim 11 wherein the donor monoclonal antibody is RSV20.

18. The composition of Claim 16 wherein the altered antibody is HuRSV19VH/VK.

19. The composition of Claim 16 wherein the altered antibody is HuRSV19VHFNS/HuRSV19VK.

20.. The composition of Claim 10 wherein the altered antibody is a Fab fragment or a $(Fab')_2$ fragment.

21. A method of preventing human RSV infection in a human in need thereof which comprises administering to such human, an effective, human RSV infection inhibiting dose of the altered antibody of any of Claims 4, 5, 6, 7, 8, and 9.

22. The method of Claim 21 wherein one dose of approximately 1 mg/kg to approximately 20 mg/kg of the altered antibody is

22. The method of Claim 21 wherein one dose of approximately 1 mg/kg to approximately 20 mg/kg of the altered antibody is administered parenterally, preferably i.v. (intravenously) or i.m. (intramuscularly).

23. The method of Claim 21 wherein one dose of approximately 200 ug/kg to approximately 2 mg/kg of the altered antibody is administered i.n. (intranasally).

24. The method of Claim 22 wherein the dose is repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April).

25. The method of Claim 23 wherein the dose is repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April).

26. The method of Claim 21 wherein, at the beginning of the RSV season, one dose of approximately 5 mg/kg to approximately 100 mg/kg of the altered antibody is administered i.v. or i.m., or one dose of approximately 0.5 mg/kg to approximately 10 mg/kg of such antibody is administered i.n

27. The method of Claim 21 wherein the altered antibody is HuRSV19VHFNS/HuRSV19VK.

28. The method of Claim 21 wherein the altered antibody is HuRSV19VH/VK.

29. A method of therapeutically treating human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection therapeutic dose of the altered antibody of any of Claims 4, 5, 6, 7, 8, and 9.

30. The method of Claim 29 wherein one dose of approximately 2 mg/kg to approximately 20 mg/kg of the altered antibody is administered parenterally., preferably i.v. or i.m.

31. The method of Claim 29 wherein approximately 200 ug/kg to approximately 2 mg/kg of the altered antibody is administered i.n.

32. A monoclonal antibody generated against Epitope 417-438.

33. A pharmaceutical composition comprising the monoclonal antibody of Claim 32 and a pharmaceutically acceptable carrier or diluent.

34. A method of preventing human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection inhibiting dose of the monoclonal antibody of Claim 32.

35. The method of Claim 34 wherein one dose of approximately 1 mg/kg to approximately 20 mg/kg of the monoclonal antibody is administered parenterally, preferably i.v. (intravenously) or i.m. (intramuscularly).

36. The method of Claim 34 wherein one dose of approximately 200 ug/kg to approximately 2 mg/kg of the monoclonal antibody is administered i.n. (intranasally).

37. The method of Claim 35 wherein such dose is repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April).

38. The method of Claim 36 wherein such dose is repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April).

39. The method of Claim 34 wherein, at the beginning of the RSV season, one dose of approximately 5 mg/kg to approximately 100 mg/kg of the monoclonal antibody is administered i.v. or i.m., or one dose of approximately 0.5 mg/kg to approximately 10 mg/kg of such antibody is administered i.n.

40. A method of therapeutically treating human RSV infection in a human in need thereof which comprises administering to such human an effective, human RSV infection therapeutic dose of the monoclonal antibody of Claim 32.

41. The method of Claim 40 wherein one dose of approximately 2 mg/kg to approximately 20 mg/kg of the monoclonal antibody is administered parenterally, preferably i.v. or i.m.

42. The method of Claim 40 wherein approximately 200 ug/kg to approximately 2 mg/kg of the antibody is administered i.n.

43. The monoclonal antibody of Claim 32 which is a Fab fragment.

44. The method of Claim 34 wherein the monoclonal antibody is administered by inhalation.

45. The method of Claim 40 wherein the monoclonal antibody is administered by inhalation.

46. The method of Claim 21 wherein the altered antibody is administered by inhalation.

47. The method of Claim 29 wherein the altered antibody is administered by inhalation.

48. A method for effecting minimal modifications within the variable region frameworks of an altered antibody necessary to produce an altered antibody with increased binding affinity comprising the following steps:

(a) analysis of framework amino acids known to be critical for interaction with CDRs, and production and testing of altered antibodies where single framework amino acids have been substituted by the corresponding amino acids from the same source as the CDRs;

(b) analysis of framework amino acids adjacent to CDRs, and production and testing of altered antibodies where one or more of the amino acids within 4 residues of CDRs have been substituted by the corresponding amino acids from the same source of the CDRs;

(c) analysis of framework residues within the altered antibody, and production and testing of altered antibodies where single amino acids have been substituted by the corresponding amino acids with major differences in charge, size or hydrophobicity from the same source of CDRs.

49. A recombinant plasmid containing the coding sequence of the altered antibody of Claim 1.

50. A mammalian cell-line transfected with the recombinant plasmid of Claim 49.

51. A recombinant plasmid containing the coding sequence of the monoclonal antibody of Claim 32.

52. A mammalian cell line transfected with the recombinant plasmid of Claim 51.

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 q  v  q  l  q  e/q  s  g  t  e  l  e  r  s  g  a  s  v  k  l  s  c  t  a  s  g
CAGGTCCAGCTGCAGSAGTCWGGGACAGAGCTTGAGAGGTCAGGGGCCTCAGTCAAGTTGTCCTGCACAGCTTCTGGC
                      1
 f  n  i  k [d  y  y  m  h] w  m  k  q  r  p  d  q  g  l  e  w  i  g [w  i  d
TTCAACATTAAAGACTACTATATGCACTGGATGAAGCAGAGGCCTGACCAGGGCCTGGAGTGGATTGGATGGATTGA
          2
 p  e  n  d  d  v  q  y  a  p  k  f  q  g] k  a  t  m  t  a  d  t  s  s  n  t
TCCTGAGAATGATGATGTTCAATATGCCCCGAAGTTCCAGGGCAAGGCCACTATGACTGCAGACACGTCCTCCAACAC
                                                                  3
 a  y  l  q  l  t  s  l  t  f  e  d  t  a  v  y  f  c  n  s [w  g  s  d  f  d
AGCCTACCTGCAGCTCACCAGCCTGACATTTGAGGACACTGCCGTCTATTTCTGTAATTCATGGGGGAGTGACTTTGA
 h] w  g  q  g  t  t  v  t  v  s  s
CCACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
```

FIG. 1

```
d i q l t q s p l s l p v t l g d q a s i s c r s s q
GACATTCAGCTGACCCAGTCTCCACTCTCCCTGCCTGTCACTCTTGGAGATCAAGCCTCCATCTCTTGCAGATCTAGTCAG

                1
t l v h t d g n t y l e w f l q k p g q s p k l l i y
ACCCTTGTACATACTGATGGAAACACCTATTTAGAATGGTTTCTGCAGAAACCAGGCCAGTCTCCAAAGCTCCTGATCTAC

          2
r v s n r f s g v p d r f s g s g s g t d f t l k i s
AGAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGC

                                    3
r v e a e d l g y y f c f q g s h l p r t f g g g t k
AGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTTTCAAGGTTCACATCTTCCTCGGACGTTCGGTGGAGGGACCAAG

l e i
CTGGAGATCTAAC
```

FIG. 2

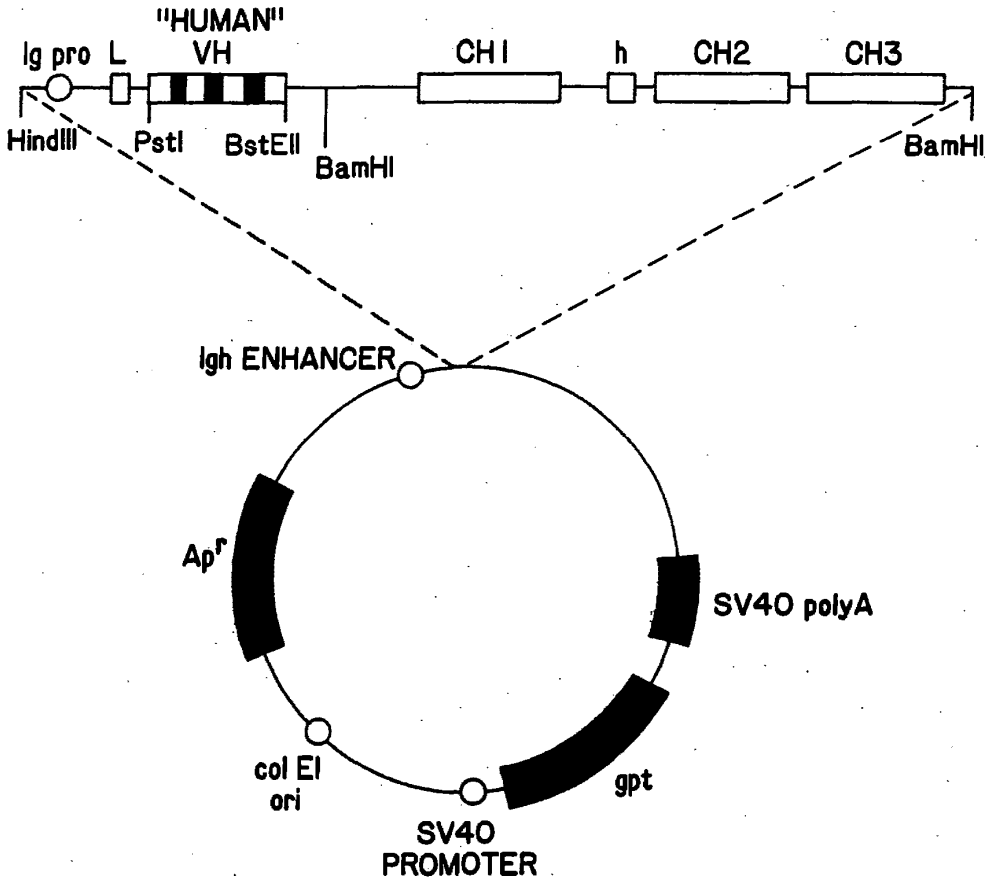
"HUMAN"
Ig pro
L
VH
CH1
h
CH2
CH3
HindIII
PstI
BstEII
BamHI
BamHI
Igh ENHANCER
Ap^r
SV40 polyA
col EI
ori
gpt
SV40
PROMOTER

FIG. 3

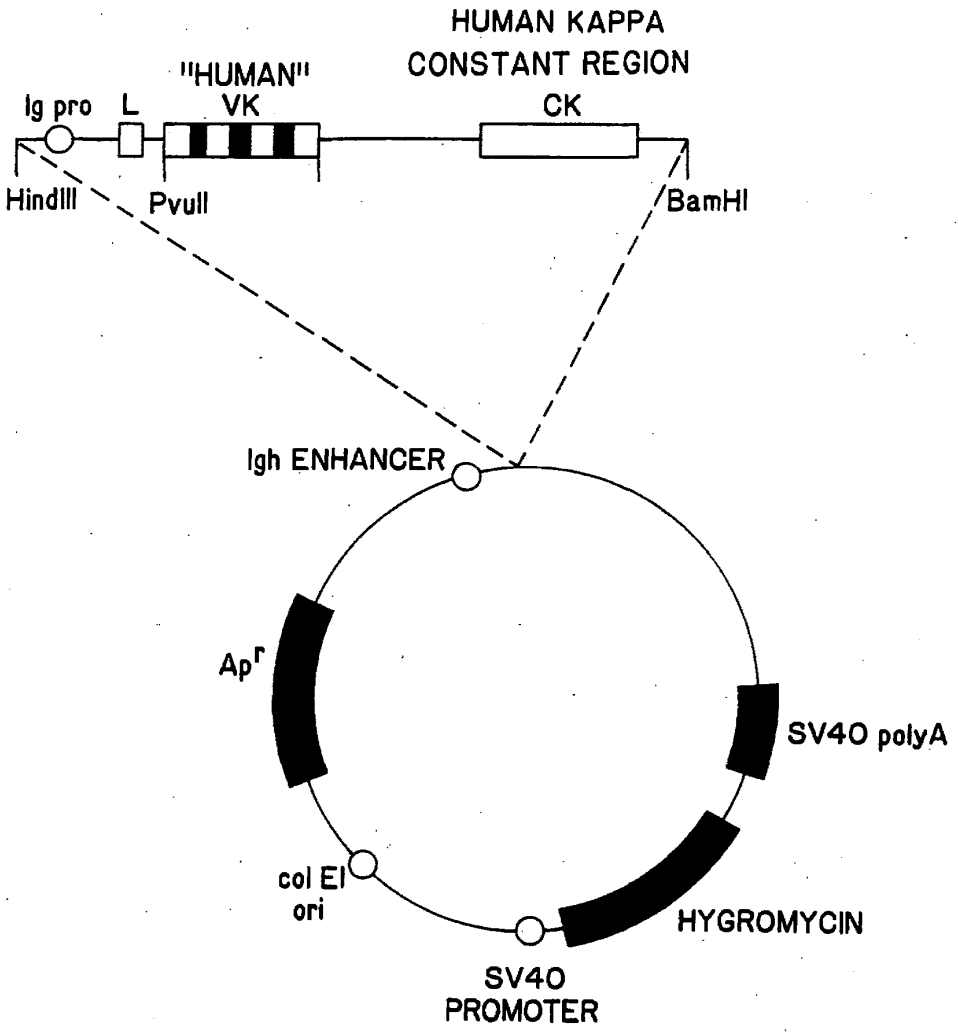

FIG. 4

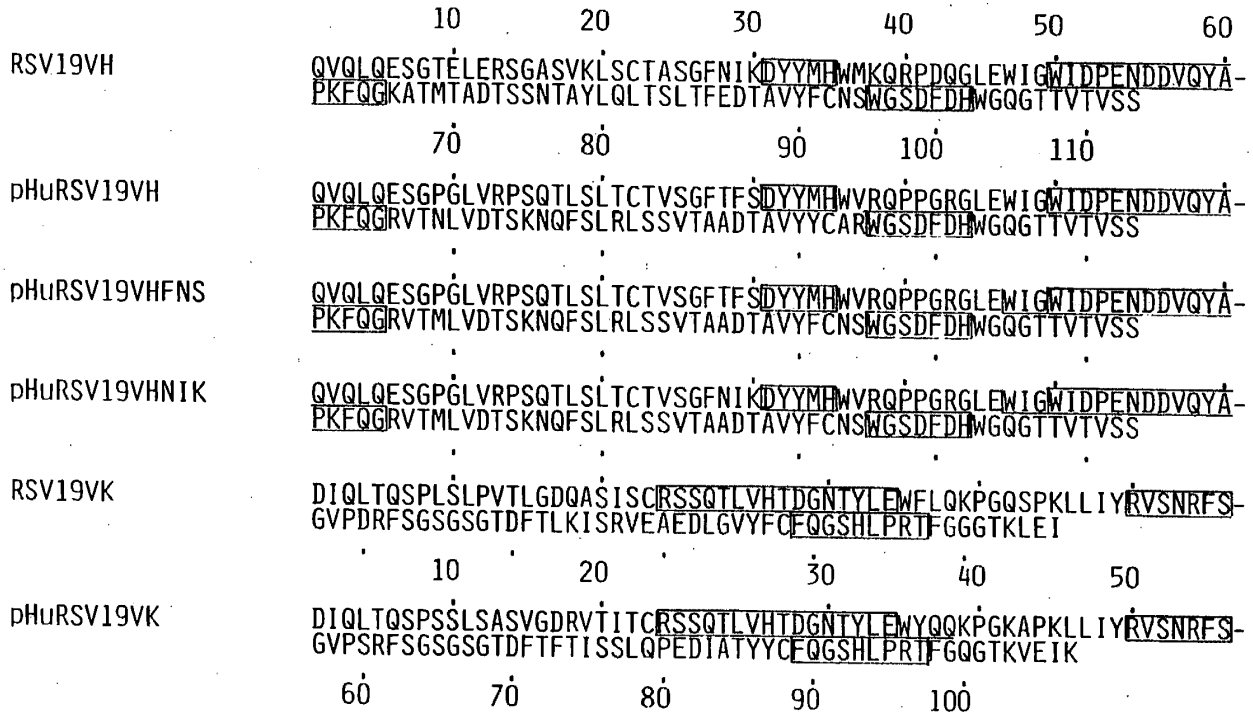

FIG. 5

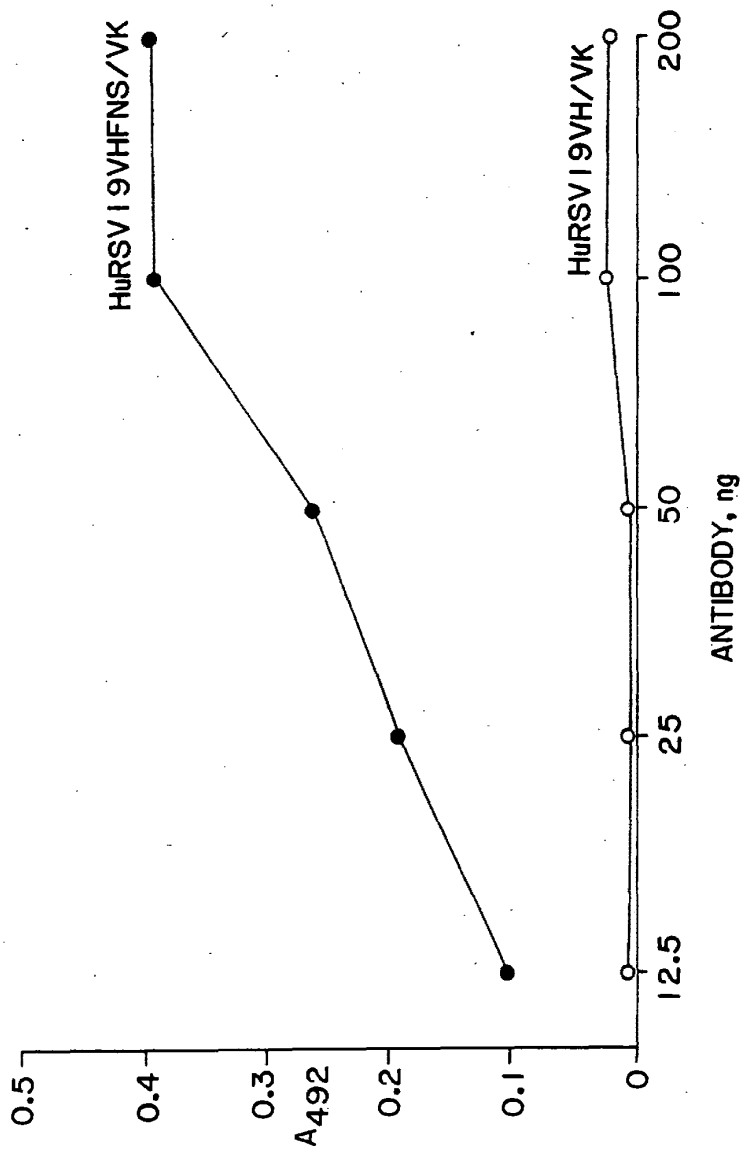

FIG. 6

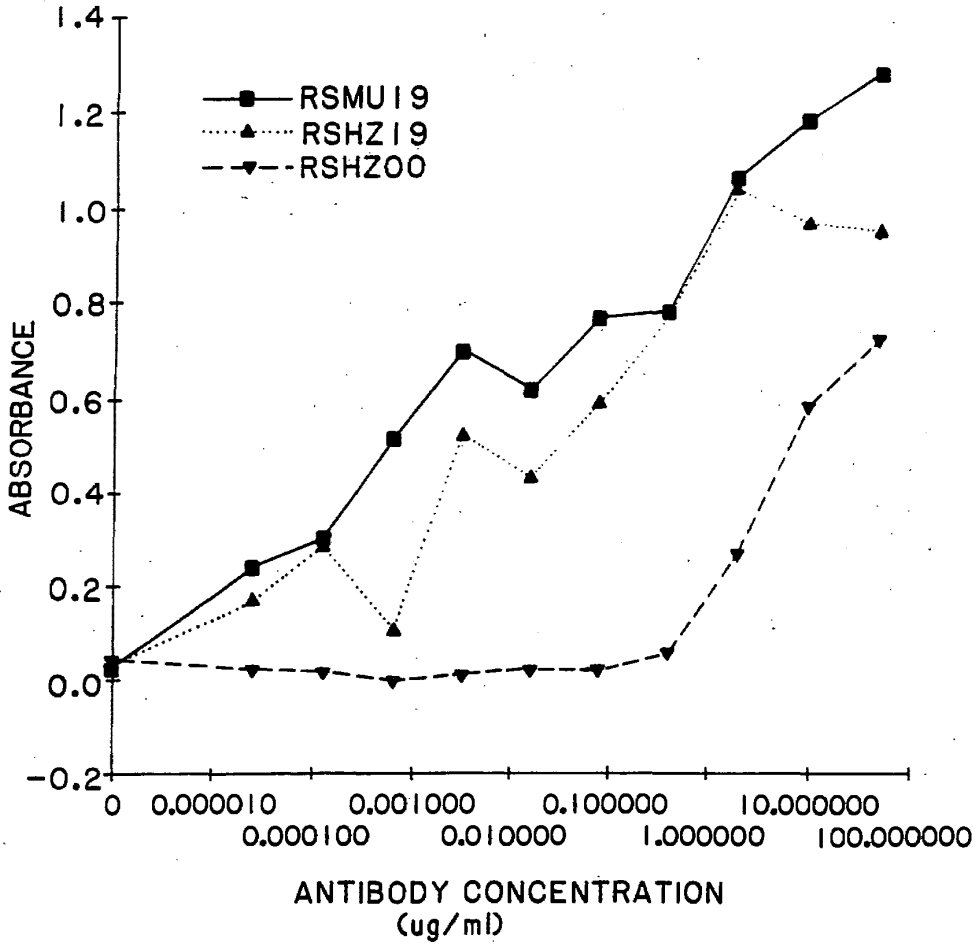

FIG. 6A

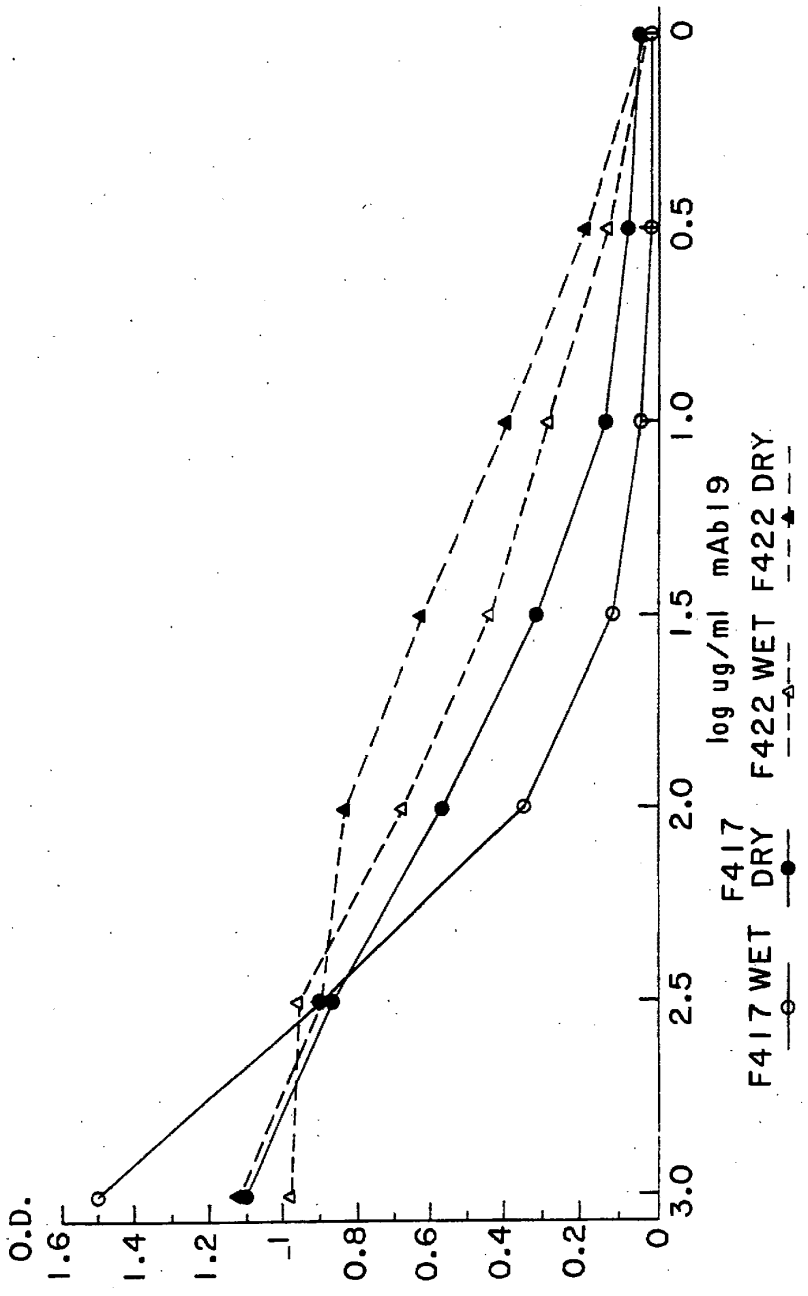

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01554

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

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

Int.Cl.5 C 07 K 15/28 C 12 P 21/08 A 61 K 39/42
C 12 N 15/09 C 12 N 5/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 P A 61 K

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

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	Proceedings of the National Academy of Sciences of USA, volume 86, no. 24, December 1989, Washington, DC, US, C. Queen et al.: "A humanized antibody that binds to the interleukin 2 receptor", pages 10029-10033, see abstract (cited in the application) ---	48
Y	---	1-21,27-29,32-34,40,43,49-52
Y	Canadian Journal of Microbiology, volume 32, no. 1, January 1986, (Ottawa, CA) M. Trudel et al.: "Immunovirological studies on human respiratory syncytial virus structural proteins", pages 15-21, see the whole article --- -/-	1-21,27-29,32-34,40,43,49-52

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09-12-1991	22.01.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme. M. van der Drift

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

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

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	US,A,4800078 (G. PRINCE et al.) 24 January 1989, see claim 1	11-21, 27-29, 33,34, 40,49-52
X,P	Biotechnology, volume 9, no. 5, May 1991 (New York, US) J. Hodgson et al.: "Making monoclonals in microbes", pages 421-425, see page 422, left-hand column, line 45 - middle column, line 54; page 423, right-hand column, lines 9-44	1-21,27-29,32-34,40, 43,48-52
A	Protein Engineering, volume 2, no. 3, September 1988 (Oxford, GB) J. Cheetham: "Reshaping the antibody combining site by CDR replacement-tailoring or tinkering to fit?", pages 170-172, see page 172, left-hand column, line 21 - right-hand column, line 19	48

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

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

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

1. [X] Claim numbers 22-26,30,31,35-39,41,42, 44-47 because they relate to subject matter not required to be searched by this Authority, namely: Please see PCT Rule 39.1(iv)

*Remark: Although claims 21,27-29,34 and 40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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

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

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

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

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

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

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9101554 SA 51153

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 23/12/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4800078	24-01-89	None	

EPO FORM P0479

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

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(22) International Filing Date: 16 September 1991 (16.09.91)

(30) Priority data:
9020282.1 17 September 1990 (17.09.90) GB

(71)(72) Applicants and Inventors: GORMAN, Scott, David [US/GB]; Flat 22, Abington House, Adrian Way, Long Road, Cambridge CB2 2SA (GB). CLARK, Michael, Ronald [GB/GB]; 108 York Street, Cambridge CB1 2PY (GB). COBBOLD, Stephen, Paul [GB/GB]; Lower Flat, 22 Guest Road, Cambridge CB1 2AL (GB). WALDMANN, Herman [GB/GB]; 11 Gurney Way, Cambridge CB4 2ED (GB).

(74) Agent: MARCHANT, James, Ian; Elkington and Fife, Prospect House, 8 Pembroke Road, Sevenoaks, Kent TN13 1XR (GB).

(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, KR, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: FRAMEWORK MUTATED ANTIBODIES AND THEIR PREPARATION

(57) Abstract

An altered antibody chain is produced in which the CDR's of the variable domain of the chain are derived from a first mammalian species. The framework-encoding regions of DNA encoding the variable domain of the first species are mutated so that the mutated framework-encoding regions encode a framework derived from a second different mammalian species. The or each constant domain of the antibody chain, if present, are also derived from the second mammalian species. An antibody which is capable of binding to human CD4 antigen is also provided together with a pharmaceutical composition comprising the antibody.

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+ **Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.**

FRAMEWORK MUTATED ANTIBODIES AND THEIR PREPARATION

The present invention relates to altered antibodies and their preparation. The invention is typically applicable to the production of humanised antibodies.

Antibodies typically comprise two heavy chains linked together by disulphide bonds and two light chains. Each light chain is linked to a respective heavy chain by disulphide bonds. 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 light chain variable domain is aligned with the variable domain of the heavy chain. The light chain constant domain is 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 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 a framework of four regions, whose sequences are relatively conserved, connected by three complementarity determining regions (CDRs). The four framework regions largely adopt a beta-sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the beta-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.

The preparation of an altered antibody in which the CDRs are derived from a different species than the framework of the antibody's variable domains is disclosed in EP-A-0239400. The CDRs may be derived from a rat or mouse monoclonal antibody. The framework of the variable domains, and the constant domains, of the altered antibody may be derived from a human antibody. Such a humanised

antibody elicits a negligible immune response when administered to a human compared to the immune response mounted by a human against a rat or mouse antibody. Humanised CAMPATH-1 antibody is disclosed in EP-A-0328404.

We have now devised a new way of preparing an altered antibody. In contrast to previous proposals, this involves altering the framework of a variable domain rather than the CDRs. This approach has the advantages that it does not require a pre-existing cDNA encoding, for example, a human framework to which to reshape and that it is technically easier than prior methodologies.

Accordingly, the present invention provides a process for the preparation of an antibody chain in which the CDRs of the variable domain of the antibody chain are derived from a first mammalian species and the framework of the variable domain and, if present, the or each constant domain of the antibody chain are derived from a second different mammalian species, which process comprises:

(i) mutating the framework-encoding regions of DNA encoding a variable domain of an antibody chain of the said first species such that the mutated framework-encoding regions encode the said framework derived from the said second species; and

(ii) expressing the said antibody chain utilising the mutated DNA from step (i).

A variable domain of either or both chains of an antibody can therefore be altered by:

(a) determining the nucleotide and predicted amino acid sequence of a variable domain of a selected antibody chain of the said first species;

(b) determining the antibody framework to which the framework of the said variable domain is to be altered;

(c) mutating the framework-encoding regions of DNA encoding the said variable domain such that the mutated

framework-encoding regions encode the framework determined upon in step (b);

(d) linking the mutated DNA obtained in step (c) to DNA encoding a constant domain of the said second species and cloning the DNA into an expression vector; and

(e) introducing the expression vector into a compatible host cell and culturing the host cell under such conditions that antibody chain is expressed.

The antibody chain may be co-expressed with a complementary antibody chain. At least the framework of the variable domain and the or each constant domain of the complementary chain generally are derived from the said second species also. A light chain and a heavy chain may be co-expressed. Either or both chains may have been prepared by the process of the invention. Preferably the CDRs of both chains are derived from the same selected antibody. An antibody comprising both expressed chains can be recovered.

The antibody preferably has the structure of a natural antibody or a fragment thereof. The antibody may therefore comprise a complete antibody, a $(Fab')_2$ fragment, a Fab fragment, a light chain dimer or a heavy chain. The antibody may be an IgG such as an IgG1, IgG2, IgG3 or IgG4 IgM, IgA, IgE or IgD. Alternatively, the antibody may be a chimaeric antibody of the type described in WO 86/01533.

A chimaeric antibody according to WO 86/01533 comprises an antigen binding region and a non-immunoglobulin region. The antigen binding region is an antibody light chain variable domain or heavy chain variable domain. Typically, the chimaeric antibody comprises both light and heavy chain variable domains. The non-immunoglobulin region is fused at its C-terminus to the antigen binding region. The non-immunoglobulin region is typically a non-immunoglobulin protein and may be an enzyme region, a region derived from a protein having known binding specificity, from a protein

toxin or indeed from any protein expressed by a gene. The two regions of the chimaeric antibody may be connected via a cleavable linker sequence.

The invention is preferably employed to humanise an antibody, typically a monoclonal antibody and, for example, a rat or mouse antibody. The framework and constant domains of the resulting antibody are therefore human framework and constant domains whilst the CDRs of the light and/or heavy chain of the antibody are rat or mouse CDRs. Preferably all CDRs are rat or mouse CDRs. The antibody may be a human IgG such as IgG1, IgG2, IgG3, IgG4; IgM; IgA; IgE or IgD carrying rat or mouse CDRs.

The process of the invention is carried out in such a way that the resulting antibody retains the antigen binding capability of the antibody from which it is derived. An antibody is reshaped according to the invention by mutating the framework-encoding regions of DNA coding for the variable domains of the antibody. This antibody and the reshaped antibody should both be capable of binding to the same antigen.

The starting antibody is typically an antibody of a selected specificity. In order to ensure that this specificity is retained, the variable domain framework of the antibody is preferably reshaped to about the closest variable domain framework of an antibody of another species. By "about the closest" is meant about the most homologous in terms of amino acid sequences. Preferably there is a homology of at least 50% between the two variable domains.

There are four general steps to reshape a monoclonal antibody. These are:

(1) determining the nucleotide and predicted amino acid sequence of the starting antibody light and heavy chain variable domains;

(2) designing the reshaped antibody, i.e. deciding which antibody framework region to use during the reshaping process;

(3) the actual reshaping methodologies/techniques; and

(4) the transfection and expression of the reshaped antibody.

These four steps are explained below in the context of humanising an antibody. However, they may equally well be applied when reshaping to an antibody of a non-human species.

Step 1: Determining the nucleotide and predicted amino acid sequence of the antibody light and heavy chain variable domains

To reshape an antibody only the amino acid sequence of antibody's heavy and light chain variable domains needs to be known. The sequence of the constant domains is irrelevant because these do not contribute to the reshaping strategy. The simplest method of determining an antibody's variable domain amino acid sequence is from cloned cDNA encoding the heavy and light chain variable domain.

There are two general methods for cloning a given antibody's heavy and light chain variable domain cDNAs: (1) via a conventional cDNA library, or (2) via the polymerase chain reaction (PCR). Both of these methods are widely known. Given the nucleotide sequence of the cDNAs, it is a simple matter to translate this information into the predicted amino acid sequence of the antibody variable domains.

Step 2: Designing the reshaped antibody

There are several factors to consider in deciding which human antibody sequence to use during the reshaping. The reshaping of light and heavy chains are considered independently of one another, but the reasoning is basically similar for each.

This selection process is based on the following rationale: A given antibody's antigen specificity and affinity is primarily determined by the amino acid sequence of the variable region CDRs. Variable domain framework residues have little or no direct contribution. The primary function of the framework regions is to hold the CDRs in their proper spacial orientation to recognize antigen. Thus the substitution of rodent CDRs into a human variable domain framework is most likely to result in retention of their correct spacial orientation if the human variable domain is highly homologous to the rodent variable domain from which they originated. A human variable domain should preferably be chosen therefore that is highly homologous to the rodent variable domain(s).

A suitable human antibody variable domain sequence can be selected as follows:

1. Using a computer program, search all available protein (and DNA) databases for those human antibody variable domain sequences that are most homologous to the rodent antibody variable domains. This can be easily accomplished with a program called FASTA but other suitable programs are available. The output of a suitable program is a list of sequences most homologous to the rodent antibody, the percent homology to each sequence, and an alignment of each sequence to the rodent sequence. This is done independently for both the heavy and light chain variable domain sequences. The above analyses are more easily accomplished if customized sub-databases are first created that only include human immunoglobulin sequences. This has two benefits. First, the actual computational time is greatly reduced because analyses are restricted to only those sequences of interest rather than all the sequences in the databases. The second benefit is that, by restricting analyses to only human immunoglobulin

sequences, the output will not be cluttered by the presence of rodent immunoglobulin sequences. There are far more rodent immunoglobulin sequences in databases than there are human.

2. List the human antibody variable domain sequences that have the most overall homology to the rodent antibody variable domain (from above). Do not make a distinction between homology within the framework regions and CDRs. Consider the overall homology.
3. Eliminate from consideration those human sequences that have CDRs that are a different length than those of the rodent CDRs. This rule does not apply to CDR 3, because the length of this CDR is normally quite variable. Also, there are sometimes no or very few human sequences that have the same CDR lengths as that of the rodent antibody. If this is the case, this rule can be loosened, and human sequences with one or more differences in CDR length can be allowed.
4. From the remaining human variable domains, the one is selected that is most homologous to that of the rodent.
5. The actual reshaped antibody (the end result) should contain CDRs derived from the rodent antibody and a variable domain framework from the human antibody chosen above.

Step 3: The actual reshaping methodologies/techniques

A cDNA encoding the desired reshaped antibody is preferably made beginning with the rodent cDNA from which the rodent antibody variable domain sequence(s) was originally determined. The rodent variable domain amino acid sequence is compared to that of the chosen human antibody variable domain sequence. The residues in the rodent variable domain framework are marked that need to be changed to the corresponding residue in the human to make the rodent framework identical to that of the human

framework. There may also be residues that need adding to or deleting from the rodent framework sequence to make it identical to that of the human.

Oligonucleotides are synthesised that can be used to mutagenize the rodent variable domain framework to contain the desired residues. Those oligonucleotides can be of any convenient size. One is normally only limited in length by the capabilities of the particular synthesizer one has available. The method of oligonucleotide-directed in vitro mutagenesis is well known.

The advantages of this method of reshaping as opposed to splicing CDRs into a human framework are that (1) this method does not require a pre-existing cDNA encoding the human framework to which to reshape and (2) splicing CDRs is technically more difficult because there is usually a large region of poor homology between the mutagenic oligonucleotide and the human antibody variable domain. This is not so much a problem with the method of splicing human framework residues onto a rodent variable domain because there is no need for a pre-existing cDNA encoding the human variable domain. The method starts instead with the rodent cDNA sequence. Also, splicing framework regions is technically easier because there is a high degree of homology between the mutagenic oligonucleotide and the rodent variable domain framework. This is true because a human antibody variable domain framework has been selected that is most homologous to that of the rodent.

The advantage of the present method of reshaping as opposed to synthesizing the entire reshaped version from scratch is that it is technically easier. Synthesizing a reshaped variable domain from scratch requires several more oligonucleotides, several days more work, and technical difficulties are more likely to arise.

Step 4: The transfection and expression of the reshaped antibody

Following the mutagenesis reactions to reshape the antibody, the cDNAs are linked to the appropriate DNA encoding light or heavy chain constant region, cloned into an expression vector, and transfected into mammalian cells. These steps can be carried out in routine fashion. A reshaped antibody may therefore be prepared by a process 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.

Preferably the DNA sequence in step a) encodes both the variable domain and the or each constant domain of the antibody chain, the or each constant domain being derived from the first antibody. The antibody can be recovered and purified. The cell line which is transformed to produce the altered antibody may be a Chinese Hamster Ovary (CHO) cell line or 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) 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.

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 or other suitable method.

An antibody is consequently produced in which CDRs of a variable domain of an antibody chain are homologous with the corresponding CDRs of an antibody of a first mammalian species and in which the framework of the variable domain and the constant domains of the antibody are homologous with the corresponding framework and constant domains of an antibody of a second, different, mammalian species. Typically, all three CDRs of the variable domain of a light or heavy chain are derived from the first species.

The present process has been applied to obtain an antibody against human CD4 antigen. Accordingly, the invention also provides an antibody which is capable of binding to human CD4 antigen, in which the CDRs of the light chain of the antibody have the amino acid sequences:

CDR1: LASEDIYSDLA
CDR2: NTDTLQN
CDR3: QQYNNYPWT,

in which the CDRs of the heavy chain of the antibody have the amino acid sequences:

CDR1: NYGMA
CDR2: TISHDGSDTYFRDSVKG
CDR3: QGTIAGIRH, and

in which the framework of the variable domain and, if present, the or each constant domain of each chain are derived from a mammalian non-rat species.

The antibody preferably has the structure of a natural antibody or a fragment thereof. The antibody may therefore comprise a complete antibody, a $(Fab')_2$ fragment, a Fab fragment, a light chain dimer or a heavy chain.

The antibody may be an IgG such as IgG1, IgG2, IgG3 or IgG4 IgM, IgA, IgE or IgD. Alternatively, the antibody may be a chimaeric antibody of the type described in WO 86/01533.

A chimaeric antibody according to WO 86/01533 comprises an antigen binding region and a non-immunoglobulin region. The antigen binding region is an antibody light chain variable domain or heavy chain variable domain. Typically the chimaeric antibody comprises both light and heavy chain variable domains. The non-immunoglobulin region is fused at its C-terminus to the antigen binding region. The non-immunoglobulin region is typically a non-immunoglobulin protein and may be an enzyme region, a region derived from a protein having known binding specificity, from a protein toxin or indeed from any protein expressed by a gene. The two regions of the chimaeric antibody may be connected via a cleavable linker sequence.

The invention is preferably employed to humanise a CD4 antibody such as a rat or mouse CD4 antibody. The framework and the constant domains of the resulting antibody are therefore human framework and constant domains whilst the CDRs of the light and/or heavy chain of the antibody are rat or mouse CDRs. Preferably all CDRs are rat or mouse CDRs. The antibody may be a human IgG such as IgG1, IgG2, IgG3, IgG4; IgM; IgA; IgE or IgD carrying rat or mouse CDRs.

Preferably the framework of the antibody heavy chain is homologous to the corresponding framework of the human antibody KOL (Schmidt et al, Hoppe-Seyler's Z. Physiol. Chem., 364 713-747, 1983). The sixth residue of framework 4 in this case is suitably Thr or Pro, preferably Thr. This residue is the 121st residue in the KOL antibody heavy chain variable region (Schmidt et al, 1983), and is identified as residue 108 by Kabat (Kabat et al, "Sequences of proteins of immunological interest", US Dept of Health

and Human Services, US Government Printing Office, 1987). Alternatively, the framework of the antibody heavy chain is homologous to the corresponding framework of the human antibody NEW (Saul et al, J. Biol.Chem. 253: 585-597, 1978). The final residue of framework 1 in this case is suitably Ser or Thr, preferably Ser. This residue is at position 30 (Kabat et al, 1987). Preferably the framework of the antibody light chain is homologous to the variable domain framework of the protein REI (Epp et al, Eur. J. Biochem., 45, 513-524, 1974).

The framework regions of one or both chains of a CD4 antibody can be reshaped by the present process. Alternatively, one or both chains of a CD4 antibody may be reshaped by the procedure described in EP-A-0239400. The procedure of EP-A-0239400 involves replacing CDRs rather than the replacement of frameworks. The CDRs are grafted onto a framework derived from a mammalian non-rat species, typically a human. This may be achieved by oligonucleotide-directed in vitro mutagenesis of the CDR-encoding regions of an antibody chain, light or heavy, from a mammalian non-rat species. The oligonucleotides in such an instance are selected so that the resulting CDR-grafted antibody has the light chain CDRs 1 to 3 and the heavy chain CDRs 1 to 3 shown above.

The reshaped CD4 antibody can be used to induce tolerance to an antigen. It can be used to alleviate autoimmune diseases such as rheumatoid arthritis. It can be used to prevent graft rejection. Tolerance to a graft such as an organ graft or a bone marrow transplantation can be achieved. Also, the reshaped CD4 antibody might be used to alleviate allergies. Tolerance to allergens could be achieved.

The CD4 antibody may be depleting or non-depleting. A depleting antibody is an antibody which depletes more than

50%, for example from 90 to 99%, of target cells in vivo. A non-depleting antibody depletes fewer than 50%, for example, from 10 to 25% and preferably less than 10% of target cells in vivo. A CD4 antibody may be administered alone or may be co-administered with a non-depleting or depleting CD8 antibody. The CD4 antibody, depleting or non-depleting, and CD8 monoclonal antibody, depleting or non-depleting, may be administered sequentially in any order or may be administered simultaneously. An additional antibody, drug or protein may be administered before, during or after administration of the antibodies.

A CD4 antibody and, indeed, a CD8 antibody as appropriate are given parenterally, for example intravenously. The antibody may be administered by injection or by infusion. For this purpose the antibody is formulated in a pharmaceutical composition further comprising a pharmaceutically acceptable carrier or diluent. Any appropriate carrier or diluent may be employed, for example phosphate-buffered saline solution.

The amount of non-depleting or depleting CD4 and, if desired, CD8 antibody administered to a patient depends upon a variety of factors including the age and weight of a patient, the condition which is being treated and the antigen(s) to which it is desired to induce tolerance. In a model mouse system from 1μg to 2mg, preferably from 400μg to 1mg, of a mAb is administered at any one time. In humans from 3 to 500mg, for example from 5 to 200mg, of antibody may be administered at any one time. Many such doses may be given over a period of several weeks, typically 3 weeks.

A foreign antigen(s) to which it is desired to induce tolerance can be administered to a host before, during, or after a course of CD4 antibody (depleting or non-depleting) and/or CD8 antibody (depleting or non-depleting). Typically, however, the antigen(s) is administered one week

after commencement of antibody administration, and is terminated three weeks before the last antibody administration.

Tolerance can therefore be induced to an antigen in a host by administering non-depleting or depleting CD4 and CD8 mAbs and, under cover of the mAbs, the antigen. A patient may be operated on surgically under cover of the non-depleting or depleting CD4 and CD8 mAbs to be given a tissue transplant such as an organ graft or a bone marrow transplant. Also, tolerance may be induced to an antigen already possessed by a subject. Long term specific tolerance can be induced to a self antigen or antigens in order to treat autoimmune disease such as multiple sclerosis or rheumatoid arthritis. The condition of a patient suffering from autoimmune disease can therefore be alleviated.

The following Example illustrates the invention. In the accompanying drawings:

Figure 1: shows the nucleotide and predicted amino acid sequence of rat CD4 antibody light chain variable region. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. Base pairs 1-269 (HindIII-PvuII) and 577-620 ([BglII/BclI]-BamHI) are part of the vector M13V_KPCR3, while base pairs 270-576 are from the PCR product of the CD4 antibody light chain variable region (V_L). CDRs (boxes) were identified by comparison to known immunological sequences (Kabat et al, "Sequences of proteins of immunological interest, US Dept of Health and Human Services, US Government Printing Office, 1987).

Figure 2: shows the nucleotide and predicted amino acid sequence of the reshaped CAMPATH-1 antibody light chain cDNA. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes.

Figure 3: shows the nucleotide and predicted amino acid sequence of the reshaped CD4 antibody light chain cDNA $CD4V_LREI$. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes.

Figure 4: shows the nucleotide and predicted amino acid sequence of rat CD4 antibody heavy chain variable region. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes. Base pairs 1-272 (HindIII-PstI) and 603-817 (BstEII-BamHI) are part of the vector $M13V_HPCR1$, while base pairs 273-602 are from the PCR product of the CD4 antibody heavy chain variable region (V_H).

Figure 5: shows the nucleotide and predicted amino acid sequence of the reshaped CAMPATH-1 antibody heavy chain cDNA. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes.

Figure 6: shows the nucleotide and predicted amino acid sequence of the reshaped CD4 antibody heavy chain cDNA $CD4V_HNEW\text{-}Thr^{30}$. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes.

Figure 7: shows the nucleotide and predicted amino acid sequence of the reshaped CD4 antibody heavy chain cDNA $CD4V_HNEW\text{-}Ser^{30}$. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes.

Figure 8: shows the heavy chain variable (V) region amino acid sequence of the human myeloma protein KOL. CDRs are identified by boxes. This sequence is taken from the Swiss-Prot protein sequence database.

Figure 9: shows the nucleotide and predicted amino acid sequence of the reshaped CD4 antibody heavy chain V region $CD4V_HKOL\text{-}Pro^{113}$. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes.

Figure 10: shows the nucleotide and predicted amino acid sequence of the reshaped CD4 antibody heavy chain V region $CD4V_HKOL\text{-}Pro^{113}$ without immunoglobulin promoter. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes.

Figure 11: shows the nucleotide and predicted amino acid sequence of the reshaped CD4 antibody heavy chain V region $CD4V_HKOL\text{-}Thr^{113}$. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes.

Figure 12: shows the nucleotide and predicted amino acid sequence of the reshaped CD4 antibody heavy chain V region $CD4V_HKOL\text{-}Thr^{113}$ without immunoglobulin promoter. The number of the first and last amino acid or nucleotide in each line is indicated in the left and right margins, respectively. CDRs are identified by boxes.

Figure 13: shows the results of an ELISA that compares the avidity of YNB46.1.8 and $CD4V_HKOL\text{-}Thr^{113}$ antibodies. The X-axis indicates the concentration (μg/ml) of YNB46.1.8 (triangles) or $CD4V_HKOL\text{-}Thr^{113}$ (circles) antibody. The Y-axis indicates the optical density at 492 nanometers.

EXAMPLE

1. MATERIALS AND METHODS

Isolation of monoclonal antibody. The rat-derived anti-human CD4 antibody, clone YNB46.1.8 (IgG_{2b}, kappa light chain serotype), was the result of fusion between a rat splenocyte and the Lou strain rat myeloma cell line Y3-Ag 1.2.3 (Galfre et al, Nature, 277: 131-133, 1979) and was selected by its binding to a rat T cell line NB2-6TG stably transfected with an expression vector containing a complementary DNA (cDNA) encoding the human CD4 antigen (Madden et al, Cell, 42: 93-104, 1985). Antibody was purified by high pressure liquid chromatography (HPLC).

Isolation of Antibody Variable Regions. cDNAs encoding the V_L and V_H regions of the CD4 antibody were isolated by a polymerase chain reaction (PCR)-based method (Orlandi et al, PNAS USA, 86: 3833-3837, 1989) with some modifications. Total RNA was isolated from hybridoma cells by the guanidine thiocyanate method (Chirgwin et al, Biochemistry, 18: 5294, 1979), and poly(A)$^+$ RNA was isolated by passage of total RNA through and elution from an oligo(dT)-cellulose column (Aviv and Leder PNAS USA 69: 1408, 1972). Poly(A)$^+$ RNA was heated at 70°C for 5 minutes and cooled on ice just prior to use. A 25μl first strand synthesis reaction consisted of 5μg poly(A)$^+$ RNA, 250 μM each dNTP, 50 mM Tris.HCl (pH 8.2 at 42°C), 10 mM $MgCl_2$, 100 mM KCl, 10 mM dithiothreitol, 23 units reverse transcriptase (Anglian Biotec, Colchester, U.K.), 3.5 pmoles of the V_L region-specific oligonucleotide primer V_K1FOR [5'-d(GTT AGA TCT CCA GCT TGG TCC C)] or the V_H region-specific primer V_H1FOR-B [5,-d(TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC)], and incubated for 5 minutes at 20°C and then 90 minutes at 42°C.

Subsequent 50 µl PCR amplifications consisted of 5 µl of the first strand synthesis reaction (unpurified), 500 µM each dNTP, 67 mM Tris-HCl (pH 8.8 at 25°C), 17 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 20 µg/ml gelatin, 5 units TAQ DNA polymerase (Koch-Light, Haverhill, U.K.), and 25 pmoles (each) of primers V_K1FOR and V_K1BACK [5'-d(GAC ATT CAG CTG ACC CAG TCT CCA)] for the V_L region or V_H1FOR-B and the mixed primer V_H1BACK [5'-d(AG GT(CG) (CA)A(GA) CTG CAG (GC)AG TC(TA) GG)] for the V_H region. Reactions were overlayed with mineral oil and subjected to 30 cycles of 1.5 minutes at 95°C (denaturation), 1.5 minutes at 37°C (V_L) or 50°C (V_H; annealing), and 3 minutes at 72°C (extension) with a Techne PHC-1 programmable cyclic reactor. The final cycle contained a 10 minute extension time.

The samples were frozen at -20°C and the mineral oil (a viscous liquid at -20°C) was removed by aspiration. The aqueous phases were thawed, and PCR products were purified by electrophoresis in 2% agarose gels, and then double digested with either PvuII and BglII (V_L) or PstI and BstEII (V_H) restriction enzymes, and cloned into the PvuII and BclI restriction sites of the vector M13V_KPCR3 (for V_L region; Orlandi et al, 1989) or the PstI and BstEII restriction sites of the vector M13V_HPCR1 (for V_H region). As described in the results, V_L region clones were first screened by hybridisation to a ^{32}P-labeled oligonucleotide probe [5'-d(GTT TCA TAA TAT TGG AGA CA)] specific for the CDR2 of the Y3-Ag 1.2.3 V_L region. V_L region clones not hybridising to this probe and V_H region clones were sequenced by the dideoxy chain termination method (Sanger et al, PNAS USA 74: 5463, 1977).

Reshaped Light Chain Variable Region and Expression Vector Construct.

The reshaped light chain was constructed by oligonucleotide-directed in vitro mutagenesis in an M13 vector by priming with three oligonucleotides simultaneously on a 748 base single-stranded cDNA template encoding the entire V_L and kappa constant (C_K) regions of the reshaped CAMPATH-1 antibody (Reichmann et al, Nature 332: 323-327, 1988). The three oligonucleotides [5'-d(AGA GTG ACC ATC ACC TGT CTA GCA AGT GAG GAC ATT TAC AGT GAT TTA GCA TGG TAC CAG CAG AAG CCA), 5'-d(CTG CTG ATC TAC AAT ACA GAT ACC TTG CAA AAT GGT GTG CCA AGC AGA TTC), 5'-d(ATC GCC ACC TAC TAC TGC CAA CAG TAT AAC AAT TAT CCG TGG ACG TTC GGC CAA GGG ACC)] were designed to replace each of the three CDRs in the REI-based human antibody V_L region framework that is part of the reshaped CAMPATH-1 antibody V_L region (Reichmann et al, 1988). A clone containing each of the three mutant oligonucleotides was identified by nucleotide sequencing and was subcloned into the HindIII site of the expression vector pHβAPr-1 (Gunning et al, PNAS, 84: 4831-4835, 1987) which also contained a dihydrofolate reductase gene (Ringold et al, J.Mol.Appl. Genet. 1: 165-175, 1981) driven by a truncated SV40 promoter.

Reshaped Heavy Chain Variable Regions Based on the Variable Region Framework of the Human Antibody NEW, and Expression Vector Constructs.

Two versions of the NEW-based reshaped heavy chain were created, CD4V_HNEW-Thr^{30} and CD4V_HNEW-Ser^{30}. The CD4V_HNEW-Thr^{30} version (Figure 6) encodes a threonine residue at position 30 while the CD4V_HNEW-Ser^{30} version (Figure 7) encodes a Ser residue at position 30. As a matter of convenience, CD4V_HNEW-Thr^{30} was created first by oligonucleotide-directed in vitro mutagenesis in the vector M13mp18 by priming with three oligonucleotides

simultaneously on a 1467 base single-stranded cDNA template (Figure 5) encoding the entire heavy chain of the reshaped CAMPATH-1 antibody (Reichmann et al, 1988). The three oligonucleotides [5'-d(TCT GGC TTC ACC TTC ACC AAC TAT GGC ATG GCC TGG GTG AGA CAG CCA CCT), 5'-d(GGT CTT GAG TGG ATT GGA ACC ATT AGT CAT GAT GGT AGT GAC ACT TAC TTT CGA GAC TCT GTG AAG GGG AGA GTG),5'-d(GTC TAT TAT TGT GCA AGA CAA GGC ACT ATA GCT GGT ATA CGT CAC TGG GGT CAA GGC AGC CTC)] were designed to replace each of the three complementarity determining regions (CDRs) in the NEW-based V_H region that is part of the reshaped CAMPATH-1 antibody (Reichmann et al, 1988). A clone (Figure 6) containing each of the three mutant oligonucleotides was identified by nucleotide sequencing. CD4V_HNEW-Ser^{30} was created second by oligonucleotide-directed in vitro mutagenesis in the vector M13mp18 by priming with a single oligonucleotide on the 1458 base single-stranded cDNA template (Figure 6) encoding CD4V_HNEW-Thr^{30}. The oligonucleotide [5'-d(GCT TCA CCT TCA GCA ACT ATG GCA T)] was designed to mutate the residue at position 30 from threonine [ACC] to serine [AGC]. A clone (Figure 7) containing this mutant oligonucleotide was identified by nucleotide sequencing. Double-stranded forms of the clones CD4V_HNEW-Thr^{30} and CD4V_HNEW-Ser^{30} were subcloned as HindIII fragments into the HindIII site of the expression vector pNH316. The vector pNH316 is a modified version of the vector pHβAPr-1 (Gunning et al, PNAS, 84: 4831-4835, 1987) which was engineered to contain a neomycin resistance gene driven by a metallothionine promoter.

Reshaped Heavy Chain Variable Regions Based on the Variable Region Framework of the Human Antibody KOL, and Expression Vector Constructs

Two versions of the KOL-based reshaped heavy chain were created, $CD4V_HKOL\text{-}Thr^{113}$ and $CD4V_HKOL\text{-}Pro^{113}$. The $CD4V_HKOL\text{-}Thr^{113}$ version encodes a threonine residue at position 113 (Figure 11) while the $CD4V_HKOL\text{-}Pro^{113}$ version encodes a proline residue at position 113 (Figure 9). As a matter of convenience, $CD4V_HKOL\text{-}Thr^{113}$ was created first by oligonucleotide-directed in vitro mutagenesis of single-stranded DNA template containing the 817 base HindIII-BamHI fragment encoding the V_H region of the rat CD4 antibody (Figure 4) cloned into M13mp18 by priming simultaneously with five oligonucleotides [5'-d(CAC TCC CAG GTC CAA CTG GTG GAG TCT GGT GGA GGC GTG GTG CAG CCT GG), 5'-d(AAG GTC CCT GAG ACT CTC CTG TTC CTC CTC TGG ATT CAT CTT CAG TAA CTA TGG CAT G), 5'-d(GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG), 5'-d(ACT ATC TCC AGA GAT AAT AGC AAA AAC ACC CTA TTC CTG CAA ATG G), 5'-d(ACA GTC TGA GGC CCG AGG ACA CGG GCG TGT ATT TCT GTG CAA GAC AAG GGA C)] which were designed to replace the rat framework regions with the human framework regions of KOL. A clone containing each of the five mutant oligonucleotides was identified by nucleotide sequencing. $CD4V_HKOL\text{-}Pro^{113}$ was created second by oligonucleotide-directed in vitro mutagenesis of single-stranded DNA template containing the 817 base HindIII-BamHI fragment encoding $CD4V_HKOL\text{-}Thr^{113}$ cloned into M13mp18 by priming with the oligonucleotide [5'-d(TGG GGC CAA GGG ACC CCC GTC ACC GTC TCC TCA)]. A clone containing this mutant oligonucleotide was identified by nucleotide sequencing.

The immunoglobulin promoters were removed from the double-stranded DNA forms of clones encoding $CD4V_HKOL\text{-}Thr^{113}$ (Figure 11) and $CD4V_HKOL\text{-}Pro^{113}$ (Figure 9) by replacing (for both versions) the first 125 bp (HindIII-NcoI) with a HindIII-NcoI oligonucleotide linker fragment

[5'-d(AGC TTT ACA GTT ACT GAG CAC ACA GGA CCT CAC) and its overlapping complement 5'-d(CAT GGT GAG GTC CTG TGT GCT CAG TAA CTG TAA)]. The resultant clones, $CD4V_HKOL\text{-}Thr^{113}$ (Figure 12) and $CD4V_HKOL\text{-}Pro^{113}$ (Figure 10), now 731 bp HindIII-BamHI fragments, were separately subcloned into the HindIII and BamHI cloning sites of the expression vector pHβAPr-1-gpt (Gunning et al, PNAS USA 76, 1373, 1987) into which had been cloned the human IgG1 constant region gene (Bruggemann et al, J.Exp.Med. 166, 1351-1361, 1987) at the BamHI site. Thus, when transfected and expressed as antibody heavy chains (see below), these reshaped V_H regions are linked to human IgG1 constant regions.

Fluorescence activated cell sorter (FACS) analysis

The relative affinities of the reshaped antibodies to bind the CD4 antigen were estimated by FACS analysis. The CD4-expressing cells used in this analysis were a cloned rat T cell line NB2-6TG stably transfected with an expression vector containing a complementary DNA (cDNA) encoding the human CD4 antigen (Maddon et al, Cell, 42, 93-104, 1985). Cells were stained with the appropriate reshaped antibody followed by fluorescein-conjugated sheep anti-human antibodies (Binding Site Ltd., Birmingham, UK). Control staining (see Table 1) consisted of no antibody present during the first stage of cell staining. Mean cellular fluorescence was determined with an Ortho FACS.

Antibody avidity analysis

The relative avidities of the rat YNB46.1.8 antibody and the reshaped $CD4V_HKOL\text{-}Thr^{113}$ antibody were estimated by an enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with soluble recombinant CD4 antigen (Byrn et al, Nature, 344: 667-670, 1990) at 50 ul/well, 10 ug/ml, and then blocked with 100 ul/well phosphate buffered saline (PBS) containing 1.0% bovine serum albumin

(BSA). Antibodies were diluted in PBS containing 0.1% BSA, and added to wells (50 ul/well) for 45 minutes at room temperature. Biotinylated CD4V_HKOL-Thr^{113} antibody (10 ul/well; 20 ug/ml final concentration) was then added to each well for an additional 45 minutes. Wells were washed with PBS containing 0.1% BSA, and then 50 ul streptavidin-biotinylated horseradish peroxidase complex (Amersham; Aylesbury, UK) diluted 1:1,000 was added to each well for 30 minutes. Wells were washed with PBS containing 0.1% BSA, and 100 ul substrate (25 mM citric acid, 50 mM disodium hydrogen phosphate, 0.1% (w/v) o-phenylene diamine, 0.04% (v/v) 30% hydrogen peroxide) was added to each well. Reactions were stopped by the addition of 50 ul/well 1.0 M sulfuric acid. Optical densities at 492 nanometers (OD_{492}) were determined with an ELISA plate reader.

Transfections.

Dihydrofolate reductase deficient chinese hamster ovary (CHO^{DHFR-}) cells (10^6/T-75 flask) were cotransfected as described (Wigler et al, PNAS USA 76, 1373, 1979) with 9μg of heavy chain construct and 1 μg of the light chain construct. Transfectants were selected in medium containing 5% dialysed foetal bovine serum for 2 to 3 weeks, and antibody-secreting clones were identified by ELISAs of conditioned media. Antibody was concentrated and purified by protein-A Sepharose (Trade Mark) column chromatography.

2. RESULTS

Cloning of Light and Heavy Chain Variable Region cDNAs.

cDNAs encoding the V_L and V_H regions from CD4 antibody-secreting hybridoma cells were isolated by PCR using primers which amplify the segment of mRNA encoding the N-

terminal region through to the J region (Orlandi et al, 1989). V_L and V_H region PCR products were subcloned into the M13-based vectors M13V_KPCR3 and M13V_HPCR1, respectively. Initial nucleotide sequence analysis of random V_L region clones revealed that most of the cDNAs encoded the V_L region of the light chain expressed by the Y3-Ag 1.2.3 rat myeloma cell line (Crowe et al, Nucleic Acid Research, 17: 7992, 1989) that was used as the fusion partner to generate the anti-CD4 hybridoma. It is likely that the expression of the Y3-Ag 1.2.3 light chain mRNA is greater than that of the CD4 antibody light chain, or the Y3-Ag 1.2.3 light chain mRNA is preferentially amplified during the PCR.

To maximize the chance of finding CD4 V_L region cDNAs, we first screened all M13 clones by hybridisation to a ^{32}P-labeled oligonucleotide probe that is complementary to the CDR 2 of Y3-Ag 1.2.3 (Crowe et al, Nucleic Acid Research, 17: 7992, 1989). Subsequent sequence analysis was restricted to M13 clones which did not contain sequence complementary to this probe. In this manner, two cDNA clones from independent PCR amplifications were identified that encoded identical V_L regions. Nucleotide sequence analysis of random V_H region PCR products revealed a single species of V_H region cDNA. Two V_H cDNA clones from independent PCR amplifications were found to contain identical sequences except that the codon of residue 14 encoded proline [CCT] in one clone while the second clone encoded leucine [CTT] at the same position.

According to Kabat et al 1987, 524 of 595 sequenced V_H regions contain a proline residue at this position, while only 6 contain leucine. We have therefore chosen the proline-encoding clone for illustration (see below). As residue 14 lies well within the first V_H framework region and not in a CDR, it is unlikely to contribute directly to antigen binding, and the ambiguity at this position did not

affect the subsequent reshaping strategy. Thus, we have not investigated this sequence ambiguity further.

The cDNA sequences and their predicted amino acid sequences are shown in Figures 1 and 4. As no additional V_L or V_H region-encoding clones were found, it was assumed that these sequences were derived from the CD4 antibody genes.

Construction of reshaped antibodies.

Our goal was to investigate the importance of selecting the appropriate human V region framework during reshaping. Two reshaping strategies were employed.

First reshaping strategy.

In the first strategy, we created a reshaped antibody that incorporated the CDRs from the rat-derived CD4 antibody and the same human V region framework sequences that we had previously successfully used for the reshaped CAMPATH-1 antibody, namely an REI-based framework for the V_L region and an NEW-based framework for the V_H region (Reichmann et al, 1988). This was accomplished by oligonucleotide-directed in vitro mutagenesis of the six CDRs of the reshaped CAMPATH-1 antibody light and heavy chain cDNAs shown in Figures 2 and 5, respectively. The resultant reshaped CD4 antibody light chain (Figure 3) is called $CD4V_LREI$. Two versions of the NEW-based reshaped CD4 antibody heavy chain were created: $CD4V_HNEW\text{-}Thr^{30}$ (Figure 6) encoding a threonine residue at position 30 (in framework 1) and $CD4V_HNEW\text{-}Ser^{30}$ (Figure 7) encoding a serine residue at position 30. These two different versions were created because the successfully reshaped CAMPATH-1 antibody heavy chain bound antigen well whether position 30 encoded a threonine or serine residue

(Reichmann et al, 1988), and we chose to test both possibilities in this case as well.

Second reshaping strategy

In the second reshaping strategy, we have reshaped the CD4 antibody V_H region to contain the V_H region framework sequences of the human antibody KOL. Of all known human antibody V_H regions, the overall amino acid sequence of the V_H region of KOL is most homologous to the rat CD4 antibody V_H region. The V_H regions of the human antibodies KOL and NEW are 66% and 42% homologous to the rat CD4 antibody V_H region, respectively.

Two versions of the KOL-based reshaped CD4 antibody heavy chain V region were created that differ by a single amino acid residue within the fourth framework region: $CD4V_HKOL\text{-}Pro^{113}$ (Figure 10) encodes a proline residue at position 113 and $CD4V_HKOL\text{-}Thr^{113}$ (Figure 12) encodes a threonine residue at position 113. $CD4V_HKOL\text{-}Pro^{113}$ is "true to form" in that its framework sequences are identical to those of the KOL antibody heavy chain V region (Figure 8).

Of all known human antibody V_L regions, the overall amino acid sequence of the V_L region of the human light chain NEW is most homologous (67%) to the rat CD4 antibody V_L region. Thus, the identical reshaped light chain, $CD4V_LREI$ (described above), that was expressed with the NEW-based reshaped CD4 antibody heavy chains $CD4V_HNEW\text{-}Thr^{30}$ and $CD4V_HNEW\text{-}Ser^{30}$, is also expressed with the KOL-based reshaped CD4 antibody heavy chains $CD4V_HKOL\text{-}Pro^{113}$ and $CD4V_HKOL\text{-}Thr^{113}$. This is advantageous because expression of the same reshaped light chain with different reshaped heavy chains allows for a direct functional comparison of each reshaped heavy chain.

To summarise, four different reshaped antibodies were created. The reshaped light chain of each antibody is

called $CD4V_LREI$. The reshaped heavy chains of the antibodies are called $CD4V_HNEW\text{-}Thr^{30}$, $CD4V_HNEW\text{-}Ser^{30}$, $CD4V_HKOL\text{-}Pro^{113}$, and $CD4V_HKOL\text{-}Thr^{113}$, respectively. Each of the reshaped heavy chains contain the same human IgG1 constant region. As each reshaped antibody contains the same reshaped light chain, the name of a reshaped antibody's heavy chain shall be used below to refer to the whole antibody (heavy and light chain combination).

Relative affinities of the reshaped antibodies

The relative affinities of the reshaped antibodies were approximated by measuring their ability to bind to CD4 antigen-expressing cells at various antibody concentrations. FACS analysis determined the mean cellular fluorescence of the stained cells (Table 1).

It is clear from this analysis that the reshaped CD4 antibodies bind to CD4 antigen to varying degrees over a broad concentration range. Consider Experiment 1 of Table 1 first. Comparing $CD4V_HKOL\text{-}Thr^{113}$ antibody to $CD4V_HNEW\text{-}Thr^{30}$ antibody, it is clear that both antibodies bind $CD4^+$ cells when compared to the control, reshaped CAMPATH-1 antibody. However, $CD4V_HKOL\text{-}Thr^{113}$ antibody binds $CD4^+$ cells with far greater affinity than $CD4V_HNEW\text{-}Thr^{30}$ antibody. The lowest concentration of $CD4V_HKOL\text{-}Thr^{113}$ antibody tested (2.5 ug/ml) gave a mean cellular fluorescence nearly equivalent to that of the highest concentration of $CD4V_HNEW\text{-}Thr^{30}$ antibody tested (168 ug/ml). Experiment 2 demonstrates that $CD4V_HNEW\text{-}Ser^{30}$ antibody may bind $CD4^+$ cells somewhat better than $CD4V_HNEW\text{-}Thr^{30}$. Only 2.5 ug/ml $CD4V_HNEW\text{-}Ser^{30}$ antibody is required to give a mean cellular fluorescence nearly equivalent to 10 ug/ml $CD4V_HNEW\text{-}Thr^{30}$ antibody. Experiment 3 demonstrates that $CD4V_HKOL\text{-}Thr^{113}$ antibody may bind $CD4^+$ cells somewhat better than $CD4V_HKOL\text{-}Pro^{113}$ antibody.

From these assays, it is clear that the KOL-based reshaped antibodies are far superior to the NEW-based reshaped antibodies with regards to affinity towards $CD4^+$ cells. Also, there is a lesser difference, if any, between $CD4V_HNEW\text{-}Thr^{30}$ antibody and $CD4V_HNEW\text{-}Ser^{30}$ antibody, and likewise between $CD4V_HKOL\text{-}Thr^{113}$ antibody and $CD4V_HKOL\text{-}Pro^{113}$ antibody. A ranking of these reshaped antibodies can thus be derived based on their relative affinities for CD4+ cells:

$CD4V_HKOL\text{-}Thr^{113} > CD4V_HKOL\text{-}Pro^{113} >> CD4V_HNEW\text{-}Ser^{30} > CD4V_HNEW\text{-}Thr^{30}$

It should be restated that each of the reshaped CD4 antibodies used in the above experiments have the identical heavy chain constant regions, and are associated with identical reshaped light chains. Thus observed differences of binding to CD4+ cells must be due to differences in their heavy chain V regions.

Relative avidities of the rat YNB46.1.8 antibody and the reshaped $CD4V_HKOL\text{-}Thr^{113}$ antibody

The relative avidities of the rat YNB46.1.8 antibody and the reshaped $CD4V_HKOL\text{-}Thr^{113}$ antibody were estimated by ELISA. In this assay, the ability of each antibody to inhibit the binding of biotinylated $CD4V_HKOL\text{-}Thr^{113}$ antibody to soluble recombinant CD4 antigen was determined. Results of an experiment are shown in Figure 13. The inhibition of binding of biotinylated $CD4V_HKOL\text{-}Thr^{113}$ antibody was linear for both the unlabeled $CD4V_HKOL\text{-}Thr^{113}$ and YNB46.1.8 antibodies near the optical density of 0.3. The concentrations of $CD4V_HKOL\text{-}Thr^{113}$ and YNB46.1.8 antibodies that give an optical density of 0.3 are 28.7 and 1.56 ug/ml, respectively. Thus the avidity of the YNB46.1.8 antibody can be estimated to be 28.7/1.56 or about 18 times better than that of $CD4V_HKOL\text{-}Thr^{113}$ antibody. It should be noted that this experiment only provides a rough approximation of relative avidities, not

affinities. The rat YNB46.1.8 antibody contains a different constant region than that of the $CD4V_HKOL\text{-}Thr^{113}$ antibody, and this could affect how well the antibodies bind CD4 antigen, irrespective of their actual affinities for CD4 antigen. The actual affinity of the reshaped antibodies for CD4 antigen may be greater, lesser, or the same as the YNB46.1.8 antibody. The other reshaped antibodies $CD4V_HKOL\text{-}Pro^{113}$, $CD4V_HNEW\text{-}Ser^{30}$, and $CD4V_HNEW\text{-}Thr^{30}$ have not yet been tested in this assay.

Table 1. Mean cellular fluorescence of $CD4^+$ cells stained with reshaped antibodies

Reshaped Antibody	Concentration (μg/ml)	Mean cellular Fluorescence
Experiment 1.		
$CD4V_HKOL\text{-}Thr^{113}$	113	578.0
$CD4V_HKOL\text{-}Thr^{113}$	40	549.0
$CD4V_HKOL\text{-}Thr^{113}$	10	301.9
$CD4V_HKOL\text{-}Thr^{113}$	2.5	100.5
$CD4V_HNEW\text{-}Thr^{30}$	168	97.0
$CD4V_HNEW\text{-}Thr^{30}$	40	40.4
$CD4V_HNEW\text{-}Thr^{30}$	10	18.7
$CD4V_HNEW\text{-}Thr^{30}$	2.5	10.9
CAMPATH-1	100	11.6
CAMPATH-1	40	9.4
CAMPATH-1	10	9.0
CAMPATH-1	2.5	8.6
CONTROL	----	9.0
Experiment 2.		
$CD4V_HNEW\text{-}Thr^{30}$	168	151.3
$CD4V_HNEW\text{-}Thr^{30}$	40	81.5
$CD4V_HNEW\text{-}Thr^{30}$	10	51.0
$CD4V_HNEW\text{-}Thr^{30}$	2.5	39.3
$CD4V_HNEW\text{-}Ser^{30}$	160	260.2
$CD4V_HNEW\text{-}Ser^{30}$	40	123.5
$CD4V_HNEW\text{-}Ser^{30}$	10	68.6
$CD4V_HNEW\text{-}Ser^{30}$	2.5	49.2
CONTROL	----	35.8
Experiment 3.		
$CD4V_HKOL\text{-}Pro^{113}$	100	594.9
$CD4V_HKOL\text{-}Pro^{113}$	40	372.0
$CD4V_HKOL\text{-}Pro^{113}$	10	137.7
$CD4V_HKOL\text{-}Pro^{113}$	2.5	48.9
$CD4V_HKOL\text{-}Thr^{113}$	100	696.7
$CD4V_HKOL\text{-}Thr^{113}$	40	631.5
$CD4V_HKOL\text{-}Thr^{113}$	10	304.1
$CD4V_HKOL\text{-}Thr^{113}$	2.5	104.0
CONTROL	----	12.3

CLAIMS

1. A process for the preparation of an antibody chain in which the complementarity determining regions (CDRs) of the variable domain of the antibody chain are derived from a first mammalian species and the framework of the variable domain and, if present, the or each constant domain of the antibody chain are derived from a second different mammalian species, which process comprises:

(i) mutating the framework-encoding regions of DNA encoding a variable domain of an antibody chain of the said first species such that the mutated framework-encoding regions encode the said framework derived from the said second species; and

(ii) expressing the said antibody chain utilising the mutated DNA from step (i).

2. A process according to claim 1, wherein the framework-encoding regions of DNA encoding the variable domain of an antibody heavy chain are mutated in step (i).

3. A process according to claim 1 or 2, wherein the framework-encoding regions of DNA encoding the variable domain of an antibody light chain are mutated in step (i).

4. A process according to any one of the preceding claims, wherein the said first species is rat or mouse.

5. A process according to any one of the preceding claims, wherein the said second species is human.

6. A process according to any one of the preceding claims, comprising:

(a) determining the nucleotide and predicted amino acid sequence of a variable domain of a selected antibody chain of the said first species;

(b) determining the antibody framework to which the framework of the said domain is to be altered;

(c) mutating the framework-encoding regions of DNA encoding the said variable domain such that the mutated framework-encoding regions encode the framework determined upon in step (b).

(d) linking the mutated DNA obtained in step (c) to DNA encoding a constant domain of the said second species and cloning the DNA into an expression vector; and

(e) introducing the expression vector into a compatible host cell and culturing the host cell under such conditions that antibody chain is expressed.

7. A process according to claim 6, in which about the most homologous framework of an antibody chain of a different species is selected in step (b) as the framework to which the framework of the said variable domain is to be altered.

8. A process according to any one of the preceding claims, wherein the antibody of the said first species is a CD4 antibody.

9. A process according to any one of the preceding claims, wherein the said antibody chain is co-expressed with a complementary antibody chain and antibody comprising the said two chains is recovered.

10. An antibody which is capable of binding to human CD4 antigen, in which the CDRs of the light chain of the antibody have the amino acid sequences:

CDR1: LASEDIYSDLA
CDR2: NTDTLQN
CDR3: QQYNNYPWT

in which the CDRs of the heavy chain of the antibody have the amino acid sequences:

CDR1: NYGMA
CDR2: TISHDGSDTYFRDSVKG
CDR3: QGTIAGIRH, and

in which the framework of the variable domain and, if present, the or each constant domain of each chain are derived from a mammalian non-rat species.

11. An antibody according to claim 10, in which the mammalian non-rat species is human.

12. An antibody according to claim 11, in which the variable domain framework of the heavy chain is homologous to the heavy chain variable domain framework of the protein KOL.

13. An antibody according to claim 12, in which the heavy chain variable region has the amino acid sequence shown in the upper line in Figure 10 or 12.

14. An antibody according to claim 11, in which the variable domain framework of the heavy chain is homologous to the heavy chain variable domain framework of the protein NEW.

15. An antibody according to claim 14, in which the heavy chain variable region has the amino acid sequence shown in the upper line of Figure 6 or 7.

16. An antibody according to any one of claims 11 to 15, in which the variable domain framework of the light

chain is homologous to the variable domain framework of the protein REI.

17. An antibody according to claim 16, in which the light chain has the amino acid sequence shown in the upper line of Figure 3.

18. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, an antibody as claimed in any one of claims 10 to 17.

FIG.1

*Hin*dIII

```
  1  AAGCTTATGAATATGCAAATCCTCTGAATCTACATGGTAAATATAGGTTTGTCTATACC  59

 60  ACAAACAGAAAAACATGAGATCACAGTTCTCTCTACAGTTACTGAGCACACAGGACCTCA 119

-19    M  G  W  S  C  I  I  L  F  L  V  A  T  A  T                -5
120  CCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTAAGGGGTGCA 179

180  CAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTTT 239

 -4          G  V  H  S  D  I  Q  L  T  Q  S  P  V  S  L  S  A   13
240  CTCTCCACAGGTGTCCACTCCGACATCCAGCTGACCCAGTCTCCAGTTTCCCTGTCTGCA 299
                                                  CDR1
 14   S  L  G  E  T  V  N  I  E  C [L  A  S  E  D  I  Y  S  D  L]  33
300  TCTCTGGGAGAAACTGTCAACATCGAATGTCTAGCAAGTGAGGACATTTACAGTGATTTA 359
```

FIG. 1 (contd.)

```
                                                                CDR 2
 34  A  W  Y  Q  Q  K  P  G  K  S  P  Q  L  L  I  Y  N  T  D  T   53
360  GCATGGTATCAGCAGAAGCCAGGGAAATCTCCTCAACTCCTGATCTATAATACAGATACC  419

 54  L  Q  N  G  V  P  S  R  F  S  G  S  G  S  G  T  Q  Y  S  L   73
420  TTGCAAAATGGGGTCCCTTCACGGTTTAGTGGCAGTGGATCTGGCACACAGTATTCTCTA  479

                                                                CDR 3
 74  K  I  N  S  L  Q  S  E  D  V  A  T  Y  F  C  Q  Q  Y  N  N   93
480  AAAATAAACAGCCTGCAATCTGAAGATGTCGCGACTTATTTCTGTCAACAATATAACAAT  539

 94  Y  P  W  T  F  G  G  G  T  K  L  E  I  K  R                 108
540  TATCCGTGGACGTTCGGTGGAGGGACCAAGCTGGAGATCAAACGTGAGTAGAATTTAAAC  599

                    BamHI
600  TTTGCTTCCTCAGTTGGATCC                                        620
```

FIG. 2

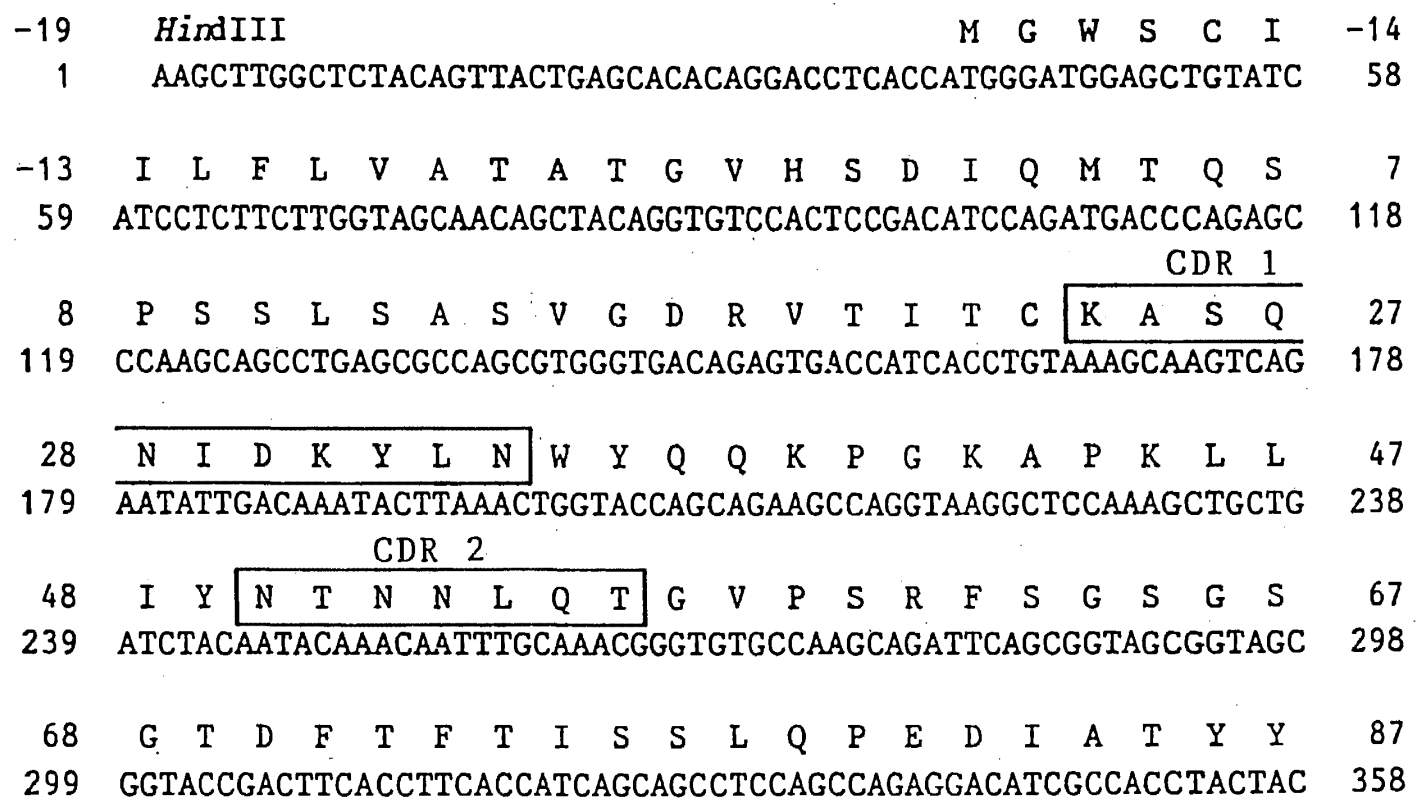
-19 HindIII M G W S C I -14
1 AAGCTTGGCTCTACAGTTACTGAGCACACAGGACCTCACCATGGGATGGAGCTGTATC 58
-13 I L F L V A T A T G V H S D I Q M T Q S 7
59 ATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCACTCCGACATCCAGATGACCCAGAGC 118
CDR 1
8 P S S L S A S V G D R V T I T C K A S Q 27
119 CCAAGCAGCCTGAGCGCCAGCGTGGGTGACAGAGTGACCATCACCTGTAAAGCAAGTCAG 178
28 N I D K Y L N W Y Q Q K P G K A P K L L 47
179 AATATTGACAAATACTTAAACTGGTACCAGCAGAAGCCAGGTAAGGCTCCAAAGCTGCTG 238
CDR 2
48 I Y N T N N L Q T G V P S R F S G S G S 67
239 ATCTACAATACAAACAATTTGCAAACGGGTGTGCCAAGCAGATTCAGCGGTAGCGGTAGC 298
68 G T D F T F T I S S L Q P E D I A T Y Y 87
299 GGTACCGACTTCACCTTCACCATCAGCAGCCTCCAGCCAGAGGACATCGCCACCTACTAC 358

FIG. 2(contd.)

CDR 3

88	C L Q H I S R P R T F G Q G T K V E I K	107
359	TGCTTGCAGCATATAAGTAGGCCGCGCACGTTCGGCCAAGGGACCAAGGTGGAAATCAAA	418
108	R T V A A P S V F I F P P S D E Q L K S	127
419	CGAACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCT	478
128	G T A S V V C L L N N F Y P R E A K V Q	147
479	GGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAG	538
148	W K V D N A L Q S G N S Q E S V T E Q D	167
539	TGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGAC	598
168	S K D S T Y S L S S T L T L S K A D Y E	187
599	AGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAG	658
188	K H K V Y A C E V T H Q G L S S P V T K	207
659	AAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG	
208	S F N R G E C Trm *Hin*dIII	214
719	AGCTTCAACAGGGGAGAGTGTTAGAAGCTT	748

FIG. 3

```
-19   HindIII                                  M  G  W  S  C  I   -14
  1   AAGCTTGGCTCTACAGTTACTGAGCACACAGGACCTCACCATGGGATGGAGCTGTATC    58

-13   I  L  F  L  V  A  T  A  T  G  V  H  S  D  I  Q  M  T  Q  S     7
 59   ATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCACTCCGACATCCAGATGACCCAGAGC  118
                                                      CDR 1
  8   P  S  S  L  S  A  S  V  G  D  R  V  T  I  T  C [L  A  S  E]   27
119   CCAAGCAGCCTGAGCGCCAGCGTGGGTGACAGAGTGACCATCACCTGTCTAGCAAGTGAG  178

 28  [D  I  Y  S  D  L  A] W  Y  Q  Q  K  P  G  K  A  P  K  L  L    47
179   GACATTTACAGTGATTTAGCATGGTACCAGCAGAAGCCAGGTAAGGCTCCAAAGCTGCTG  238
                    CDR 2
 48   I  Y [N  T  D  T  L  Q  N] G  V  P  S  R  F  S  G  S  G  S    67
239   ATCTACAATACAGATACCTTGCAAAATGGTGTGCCAAGCAGATTCAGCGGTAGCGGTAGC  298

 68   G  T  D  F  T  F  T  I  S  S  L  Q  P  E  D  I  A  T  Y  Y    87
299   GGTACCGACTTCACCTTCACCATCAGCAGCCTCCAGCCAGAGGACATCGCCACCTACTAC  358
                  CDR 3
 88   C [Q  Q  Y  N  N  Y  P  W  T] F  G  Q  G  T  K  V  E  I  K   107
359   TGCCAACAGTATAACAATTATCCGTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAA  418
```

FIG. 3(contd.)

```
108   R  T  V  A  A  P  S  V  F  I  F  P  P  S  D  E  Q  L  K  S   127
419   CGAACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCT  478

128   G  T  A  S  V  V  C  L  L  N  N  F  Y  P  R  E  A  K  V  Q   147
479   GGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAG  538

148   W  K  V  D  N  A  L  Q  S  G  N  S  Q  E  S  V  T  E  Q  D   167
539   TGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGAC  598

168   S  K  D  S  T  Y  S  L  S  S  T  L  T  L  S  K  A  D  Y  E   187
599   AGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAG  658

188   K  H  K  V  Y  A  C  E  V  T  H  Q  G  L  S  S  P  V  T  K   207
659   AAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG  718

208   S  F  N  R  G  E  C  Trm  HindIII                            214
719   AGCTTCAACAGGGGAGAGTGTTAGAAGCTT                               748
```

FIG. 4

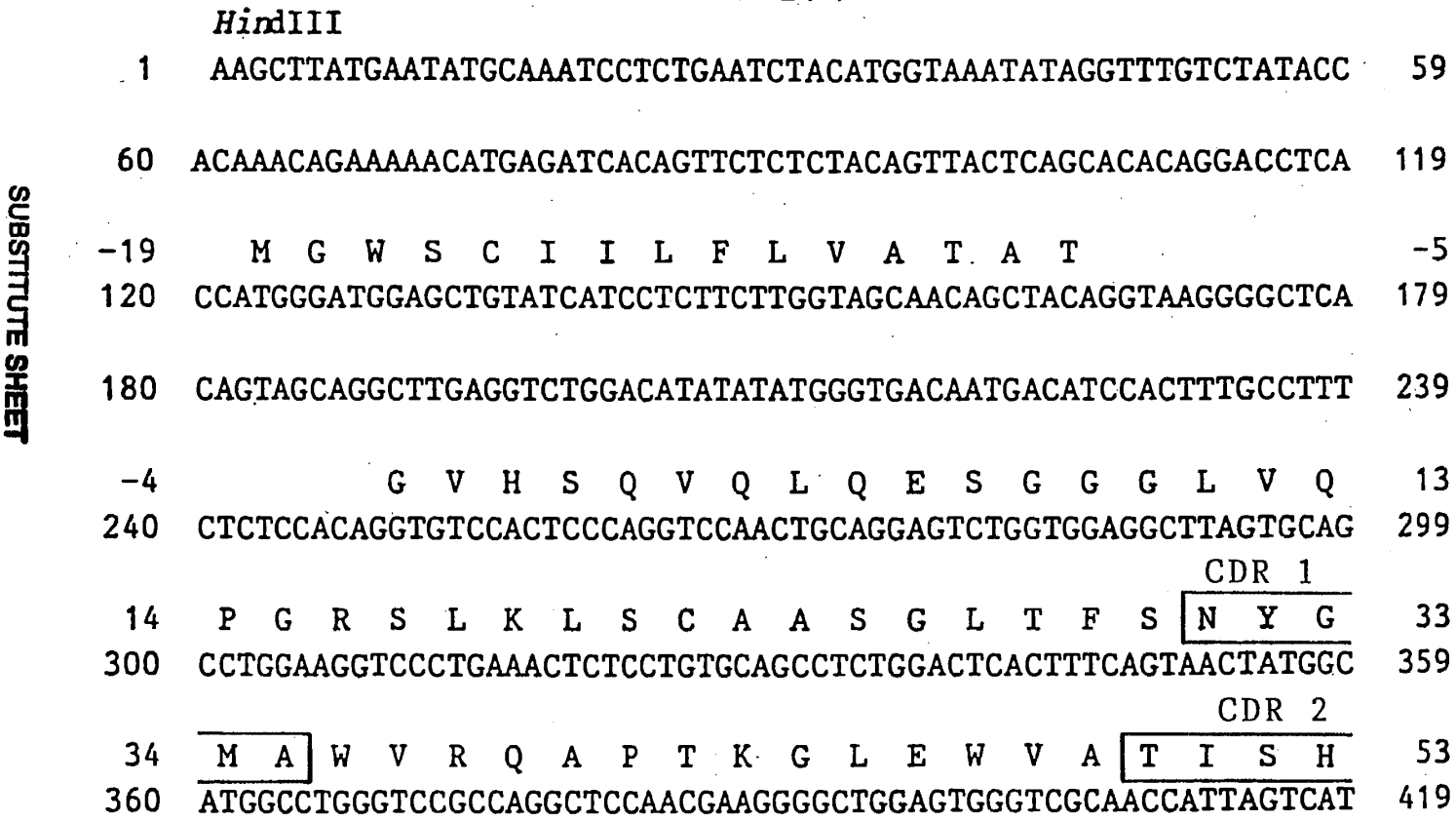
HindIII
1 AAGCTTATGAATATGCAAATCCTCTGAATCTACATGGTAAATATAGGTTTGTCTATACC 59
60 ACAAACAGAAAAACATGAGATCACAGTTCTCTCTACAGTTACTCAGCACACAGGACCTCA 119
-19 M G W S C I I L F L V A T A T -5
120 CCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTAAGGGGCTCA 179
180 CAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTTT 239
-4 G V H S Q V Q L Q E S G G G L V Q 13
240 CTCTCCACAGGTGTCCACTCCCAGGTCCAACTGCAGGAGTCTGGTGGAGGCTTAGTGCAG 299
CDR 1
14 P G R S L K L S C A A S G L T F S N Y G 33
300 CCTGGAAGGTCCCTGAAACTCTCCTGTGCAGCCTCTGGACTCACTTTCAGTAACTATGGC 359
CDR 2
34 M A W V R Q A P T K G L E W V A T I S H 53
360 ATGGCCTGGGTCCGCCAGGCTCCAACGAAGGGGCTGGAGTGGGTCGCAACCATTAGTCAT 419

FIG. 4 (contd.)

```
 54  D  G  S  D  T  Y  F  R  D  S  V  K  G  R  F  T  I  S  R  D   73
420  GATGGTAGTGACACTTACTTTCGAGACTCCGTGAAGGGCCGATTCACTATCTCCAGAGAT  479

 74  N  G  K  S  T  L  Y  L  Q  M  D  S  L  R  S  E  D  T  A  T   93
480  AATGGAAAAAGCACCCTATACCTGCAAATGGACAGTCTGAGGTCTGAGGACACGGCCACT  539

 94  Y  Y  C  A  R  Q  G  T  I  A  G  I  R  H  W  G  Q  G  T  T  113
540  TATTACTGTGCAAGACAAGGGACTATAGCAGGTATACGTCACTGGGGCCAAGGGACCACG  599

114  V  T  V  S  S                                               118
600  GTCACCGTCTCCTCAGGTGAGTCCTTACAACCTCTCTCTTCTATTCAGCTTAAATAGATT  659

660  TTACTGCATTTGTTGGGGGGGAAATGTGTGTATCTGAATTTCAGGTCATGAAGGACTAGG  719

720  GACACCTTGGGAGTCAGAAAGGGTCATTGGGAGCCCGGGCTGATGCAGACAGACATCCTC  779

                          BamHI
780  AGCTCCCAGACTTCATGGCCAGAGATTTATAGGGATCC                        817
```

FIG.5

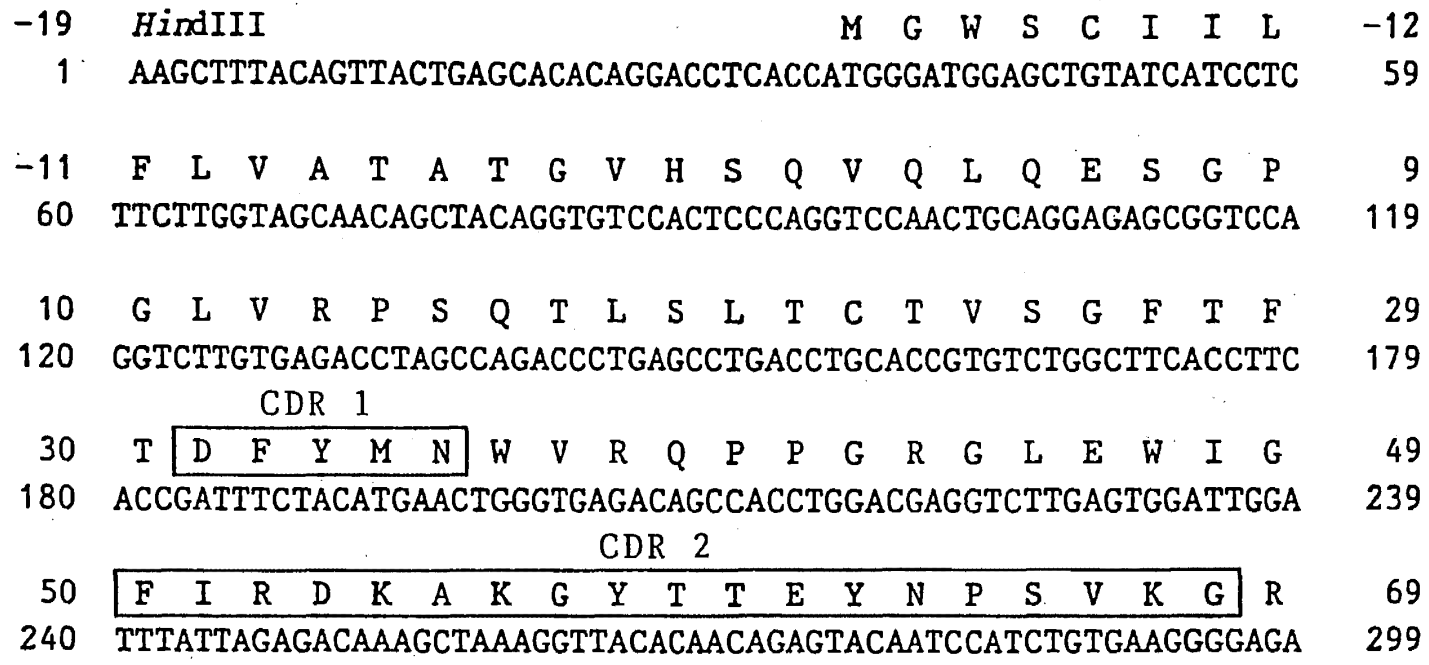
-19 HindIII M G W S C I I L -12
1 AAGCTTTACAGTTACTGAGCACACAGGACCTCACCATGGGATGGAGCTGTATCATCCTC 59
-11 F L V A T A T G V H S Q V Q L Q E S G P 9
60 TTCTTGGTAGCAACAGCTACAGGTGTCCACTCCCAGGTCCAACTGCAGGAGAGCGGTCCA 119
10 G L V R P S Q T L S L T C T V S G F T F 29
120 GGTCTTGTGAGACCTAGCCAGACCCTGAGCCTGACCTGCACCGTGTCTGGCTTCACCTTC 179
CDR 1
30 T D F Y M N W V R Q P P G R G L E W I G 49
180 ACCGATTTCTACATGAACTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGA 239
CDR 2
50 F I R D K A K G Y T T E Y N P S V K G R 69
240 TTTATTAGAGACAAAGCTAAAGGTTACACAACAGAGTACAATCCATCTGTGAAGGGGAGA 299

FIG.5 (contd.)

```
 70   V  T  M  L  V  D  T  S  K  N  Q  F  S  L  R  L  S  S  V  T    89
300   GTGACAATGCTGGTAGACACCAGCAAGAACCAGTTCAGCCTGAGACTCAGCAGCGTGACA   359
                                                  CDR 3
 90   A  A  D  T  A  V  Y  Y  C  A  R  E  G  H  T  A  A  P  F  D    109
360   GCCGCCGACACCGCGGTCTATTATTGTGCAAGAGAGGGCCACACTGCTGCTCCTTTTGAT   419

110   Y  W  G  Q  G  S  L  V  T  V  S  S  A  S  T  K  G  P  S  V    129
420   TACTGGGGTCAAGGCAGCCTCGTCACAGTCTCCTCAGCCTCCACCAAGGGCCCATCGGTC   479

130   F  P  L  A  P  S  S  K  S  T  S  G  G  T  A  A  L  G  C  L    149
480   TTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTG   539

150   V  K  D  Y  F  P  E  P  V  T  V  S  W  N  S  G  A  L  T  S    169
540   GTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGC   599
```

FIG.5 (contd.)

```
170   G  V  H  T  F  P  A  V  L  Q  S  S  G  L  Y  S  L  S  S  V    189
600   GGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTG   659

190   V  T  V  P  S  S  S  L  G  T  Q  T  Y  I  C  N  V  N  H  K    209
660   GTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAG   719

210   P  S  N  T  K  V  D  K  K  V  E  P  K  S  C  D  K  T  H  T    229
720   CCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACACA   779

230   C  P  P  C  P  A  P  E  L  L  G  G  P  S  V  F  L  F  P  P    249
780   TGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCA   839

250   K  P  K  D  T  L  M  I  S  R  T  P  E  V  T  C  V  V  V  D    269
840   AAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGAC   899
```

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FIG. 5 (contd.)

```
270   V  S  H  E  D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H   289
900   GTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCAT   959

290   N  A  K  T  K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V   309
960   AATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTC  1019

310   L  T  V  L  H  Q  D  W  L  N  G  K  E  Y  K  C  K  V  S  N   329
1020  CTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAAC  1079

330   K  A  L  P  A  P  I  E  K  T  I  S  K  A  K  G  Q  P  R  E   349
1080  AAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAA  1139

350   P  Q  V  Y  T  L  P  P  S  R  D  E  L  T  K  N  Q  V  S  L   369
1140  CCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTG  1199
```

FIG. 5(contd.)

```
370   T  C  L  V  K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G   389
1200  ACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGG  1259

390   Q  P  E  N  N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F   409
1260  CAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTC  1319

410   L  Y  S  K  L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C   429
1320  CTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGC  1379

430   S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P   448
1380  TCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCG  1439

449   G  K  Trm                 HindIII                              450
1440  GGTAAATGAGTGCGACGGCCCCAAGCTT                                   1467
```

FIG. 6

```
-19   HindIII                                  M  G  W  S  C  I  I  L    -12
  1   AAGCTTTACAGTTACTGAGCACACAGGACCTCACCATGGGATGGAGCTGTATCATCCTC      59

-11   F  L  V  A  T  A  T  G  V  H  S  Q  V  Q  L  Q  E  S  G  P       9
 60   TTCTTGGTAGCAACAGCTACAGGTGTCCACTCCCAGGTCCAACTGCAGGAGAGCGGTCCA    119

 10   G  L  V  R  P  S  Q  T  L  S  L  T  C  T  V  S  G  F  T  F     29
120   GGTCTTGTGAGACCTAGCCAGACCCTGAGCCTGACCTGCACCGTGTCTGGCTTCACCTTC    179
            CDR 1
 30   T [N  Y  G  M  A] W  V  R  Q  P  P  G  R  G  L  E  W  I  G     49
180   ACCAACTATGGCATGGCCTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGA    239
                     CDR 2
 50  [T  I  S  H  D  G  S  D  T  Y  F  R  D  S  V  K  G] R  V  T     69
240   ACCATTAGTCATGATGGTAGTGACACTTACTTTCGAGACTCTGTGAAGGGGAGAGTGACA    299
```

FIG. 6 (contd.)

```
 70   M  L  V  D  T  S  K  N  Q  F  S  L  R  L  S  S  V  T  A  A     89
300   ATGCTGGTAGACACCAGCAAGAACCAGTTCAGCCTGAGACTCAGCAGCGTGACAGCCGCC  359
                                        CDR 3
 90   D  T  A  V  Y  Y  C  A  R [Q  G  T  I  A  G  I  R  H] W  G     109
360   GACACCGCGGTCTATTATTGTGCAAGACAAGGCACTATAGCTGGTATACGTCACTGGGGT  419

110   Q  G  S  L  V  T  V  S  S  A  S  T  K  G  P  S  V  F  P  L     129
420   CAAGGCAGCCTCGTCACAGTCTCCTCAGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTG  479

130   A  P  S  S  K  S  T  S  G  G  T  A  A  L  G  C  L  V  K  D     149
480   GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGAC  539

150   Y  F  P  E  P  V  T  V  S  W  N  S  G  A  L  T  S  G  V  H     169
540   TACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCAC  599
```

FIG. 6(contd.)

```
170   T  F  P  A  V  L  Q  S  S  G  L  Y  S  L  S  S  V  V  T  V    189
600   ACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTG    659

190   P  S  S  S  L  G  T  Q  T  Y  I  C  N  V  N  H  K  P  S  N    209
660   CCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAAC    719

210   T  K  V  D  K  K  V  E  P  K  S  C  D  K  T  H  T  C  P  P    229
720   ACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCG    779

230   C  P  A  P  E  L  L  G  G  P  S  V  F  L  F  P  P  K  P  K    249
780   TGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAG    839

250   D  T  L  M  I  S  R  T  P  E  V  T  C  V  V  V  D  V  S  H    269
840   GACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCAC    899
```

FIG. 6 (contd.)

270	E D P E V K F N W Y V D G V E V H N A K	289
900	GAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAG	959
290	T K P R E E Q Y N S T Y R V V S V L T V	309
960	ACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTC	1019
310	L H Q D W L N G K E Y K C K V S N K A L	329
1020	CTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTC	1079
330	P A P I E K T I S K A K G Q P R E P Q V	349
1080	CCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTG	1139
350	Y T L P P S R D E L T K N Q V S L T C L	369
1140	TACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTG	1199

FIG. 6 (contd.)

```
 370   V  K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E     389
1200   GTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAG   1259

 390   N  N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S     409
1260   AACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGC   1319

 410   K  L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V  M     429
1320   AAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATG   1379

 430   H  E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G  K Trm   448
1380   CATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA   1439

                    HindIII
1440   GTGCGACGGCCCCAAGCTT                                            1458
```

FIG. 7

```
-19   HindIII                                  M  G  W  S  C  I  I  L    -12
  1   AAGCTTTACAGTTACTGAGCACACAGGACCTCACCATGGGATGGAGCTGTATCATCCTC      59

-11   F  L  V  A  T  A  T  G  V  H  S  Q  V  Q  L  Q  E  S  G  P       9
 60   TTCTTGGTAGCAACAGCTACAGGTGTCCACTCCCAGGTCCAACTGCAGGAGAGCGGTCCA    119

 10   G  L  V  R  P  S  Q  T  L  S  L  T  C  T  V  S  G  F  T  F     29
120   GGTCTTGTGAGACCTAGCCAGACCCTGAGCCTGACCTGCACCGTGTCTGGCTTCACCTTC    179
            CDR 1
 30   S [N  Y  G  M  A] W  V  R  Q  P  P  G  R  G  L  E  W  I  G     49
180   AGCAACTATGGCATGGCCTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGA    239
                        CDR 2
 50  [T  I  S  H  D  G  S  D  T  Y  F  R  D  S  V  K  G] R  V  T     69
240   ACCATTAGTCATGATGGTAGTGACACTTACTTTCGAGACTCTGTGAAGGGGAGAGTGACA    299
```

FIG. 7 (contd.)

```
 70   M  L  V  D  T  S  K  N  Q  F  S  L  R  L  S  S  V  T  A  A     89
300   ATGCTGGTAGACACCAGCAAGAACCAGTTCAGCCTGAGACTCAGCAGCGTGACAGCCGCC  359

                                        CDR 3
 90   D  T  A  V  Y  Y  C  A  R [Q  G  T  I  A  G  I  R  H] W  G     109
360   GACACCGCGGTCTATTATTGTGCAAGACAAGGCACTATAGCTGGTATACGTCACTGGGGT  419

110   Q  G  S  L  V  T  V  S  S  A  S  T  K  G  P  S  V  F  P  L     129
420   CAAGGCAGCCTCGTCACAGTCTCCTCAGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTG  479

130   A  P  S  S  K  S  T  S  G  G  T  A  A  L  G  C  L  V  K  D     149
480   GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGAC  539

150   Y  F  P  E  P  V  T  V  S  W  N  S  G  A  L  T  S  G  V  H     169
540   TACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCAC  599
```