**ABSTRACT**

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

82 Claims, 9 Drawing Sheets
** OTHER PUBLICATIONS **


O’Connor et al., “Calcium Dependence of an Anti-Protein C Humanized Antibody Involves Framework Residues” (manuscript).


Riechmann & Winter, “Recombinant Antibodies” (U. of London Royal Postgraduate Medical School, Wolfson Institute, Abstract) (May 1987).


Shields et al., “Inhibition of Allergic Reactions with Antibodies to IgE” International Archives of Allergy and Immunology 107(1–3):308–312 (May 1995).


* cited by examiner
FIG. 1B

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<th>40</th>
<th>50</th>
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<td>4D5</td>
<td>EVQLQQSGPELVKPGASLKLCTASGFNIKDTYIHWWKQRPEQGLEGWIGRIYPNT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HU4D5</td>
<td>EVQLVESGGGLVQPGSRLCAASGFNIKDTYIHWWRQAPGKLEWVARLYPTN</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>HUV&lt;sub&gt;H&lt;/sub&gt;III</td>
<td>EVQLVESGGGLVQPGSRLCAASGFSDYAMSWVRQAPGKLEWVAISENG</td>
<td>-------</td>
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</table>

  | 60  | 70  | 80  | 90  | 100ABC |
|---|-----|-----|-----|-----|--------|
| 4D5 | GYTRYDPKFDKATITADTSNTAYLQVSRLTEDTAVYCSRWGDGFGYAMYDVW | |||| |||| |
| HU4D5 | GYTRYADSVKGRFTISADTSKNTAYLQMSLRRAEDTAVYCSRWGDGFGYAMYDVW | || |||| |||| |
| HUV<sub>H</sub>III | SDTYYADSVKGRFTISRDDSNTLYLQMSLRRAEDTAVYCARDRGGAVSYFDVW | ------- | ---- |

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<tr>
<td>4D5</td>
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<tr>
<td>HU4D5</td>
</tr>
<tr>
<td>HUV&lt;sub&gt;H&lt;/sub&gt;III</td>
</tr>
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</table>
Anneal hu\textsubscript{V\textsc{L}} or hu\textsubscript{V\textsc{H}} oligomers to pAK1 template

1. Ligate
2. Isolate assembled oligomers
3. Anneal to pAK1 template (\textit{XhoI}\textsuperscript{−}, \textit{StuI}\textsuperscript{+})
4. Extend and ligate

---

1. Transform \textit{E. coli}
2. Isolate phagemid pool
3. Enrich for hu\textsubscript{V\textsc{L}} and hu\textsubscript{V\textsc{H}}(\textit{XhoI} \textsuperscript{+}, \textit{StuI}\textsuperscript{−})
4. Sequence verify

---

\textit{FIG. 2}
FIG. 3
**FIG. 5**

```
VL
muxCD3  DIQMTQKTSSLASLGDRVTISCRASQDIIRNYLNYWQQKP
huxCD3v1 DIQMTQSPSSLASVGDRVTITCRASQDIRNYLNYWQQKP
huki    DIQMTQSPSSLASVGDRVTITCRASQISNYLAWYQQKP

muCD3  DIQMTQKTVSSLASLGDRVTISCRASQDIIRNYLNYWQQKP
huxCD3v1 DIQMTQSPSSLASVGDRVTITCRASQDIRNYLNYWQQKP
huki    DIQMTQSPSSLASVGDRVTITCRASQISNYLAWYQQKP

VH
muxCD3  EVQLQGSPGELVKPGASMKISCKASGFTGTMNWKQ5
huxCD3v1 EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA
huki    EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA

muCD3  EVQLQGSPGELVKPGASMKISCKASGFTGTMNWKQ5
huxCD3v1 EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA
huki    EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA

CDR-L1

muCD3  DIQMTQKTSSLASLGDRVTISCRASQDIIRNYLNYWQQKP
huxCD3v1 DIQMTQSPSSLASVGDRVTITCRASQDIRNYLNYWQQKP
huki    DIQMTQSPSSLASVGDRVTITCRASQISNYLAWYQQKP

CDR-L2

muCD3  DIQMTQKTVSSLASLGDRVTISCRASQDIIRNYLNYWQQKP
huxCD3v1 DIQMTQSPSSLASVGDRVTITCRASQDIRNYLNYWQQKP
huki    DIQMTQSPSSLASVGDRVTITCRASQISNYLAWYQQKP

CDR-L3

muCD3  DIQMTQKTVSSLASLGDRVTISCRASQDIIRNYLNYWQQKP
huxCD3v1 DIQMTQSPSSLASVGDRVTITCRASQDIRNYLNYWQQKP
huki    DIQMTQSPSSLASVGDRVTITCRASQISNYLAWYQQKP

CDR-H1

muCD3  HGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAY
huxCD3v1 PGKGLEWVALNPYKGVSTYNQKFKDKATLTVDKSSSTAY
huki    PGKGLEWVALNPYKGVSTYNQKFKDKATLTVDKSSSTAY

CDR-H2

muCD3  HGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAY
huxCD3v1 PGKGLEWVALNPYKGVSTYNQKFKDKATLTVDKSSSTAY
huki    PGKGLEWVALNPYKGVSTYNQKFKDKATLTVDKSSSTAY

CDR-H3

muCD3  HGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAY
huxCD3v1 PGKGLEWVALNPYKGVSTYNQKFKDKATLTVDKSSSTAY
huki    PGKGLEWVALNPYKGVSTYNQKFKDKATLTVDKSSSTAY

FIG. 5
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<th>pH52-8.0</th>
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<td>FIG. 6A-1</td>
<td>QVQLQSPGPELVKPGASVKSCKTSGGFTE</td>
</tr>
<tr>
<td>10 20 30</td>
<td>.*** .. *** ** ***</td>
</tr>
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<td>40 50 60</td>
<td>70 80</td>
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<tr>
<td>YTMHWMKSHGKLEWIGFNPKNGGSHHQRFMDKATLAVDKSTSTAYM</td>
<td>YTMHWMRQAPPKGLEGWAGINPKNGGTSNHQRDFDRTISVSDKSTSTAYM</td>
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<tr>
<td>90 100 110 120 130</td>
<td>110 120 130 140 150</td>
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<tr>
<td>ELRSLTSEDGIYYCARWRGLNYGFDVRYFDVAGGTTVTSSASTKGPSS</td>
<td>QMNLSRAEDTAVYCARWRGLNYGFDVRYFDVQGTLTVTSSASTKGPS</td>
</tr>
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<td>.** .*** ******** ** ************</td>
<td>110 120 130 140 150</td>
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<td>140 150 160 170 180</td>
<td>140 150 160 170 180</td>
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<tr>
<td>VFPAPSSKSTGGAALGLCLVTDYFPEPVTWNASGALTGGTHTFPAVL</td>
<td>VFPAPCSRTSSEASTAALGLVTDYFPEPVTWNASGALTGGTHTFPAVL</td>
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<tr>
<td>160 170 180</td>
<td>190 200</td>
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<td>QGGLYSSSVVTTPSSSLGQTYICNVRHNTKVDKKEPKSCKTH</td>
<td>QGGLYYSSSVVTTPSSSNFQTYTCNVHDHNTKIDKTVKCC---V</td>
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<td>190 200 210 220 230</td>
<td>210 220 230 240</td>
</tr>
<tr>
<td>240 250 260 270 280</td>
<td>250 260 270 280 290</td>
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<tr>
<td>TCPPCPAPPELLGGPSVFLFPPKDTLMISRTPEVTCVVVDVSHEDEPEVK</td>
<td>ECPAPPPAP-VAGPSVFLFPPKDTLMISRTPEVTCVVVDVSHEDEPEVQ</td>
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**FIG. 6A-2**

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<tr>
<th>pH52H4-160</th>
<th>FNWYVDGVEVHNAKTQPREEQYNSTYRVSVLTVLHQDWLNGKEYKCKVS</th>
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<tr>
<td>pH52-8.0</td>
<td>FNWYVDGMEVHNAKTQPREQFNSTFRVSVLTVVHQDWLNGKEYKCKVS</td>
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<table>
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<tr>
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<th>NKALPAPIEKTIKAGQPREEQVIMTSQVSLTCLVKGFYIP</th>
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<tr>
<td>pH52-8.0</td>
<td>NKGLPAPIEKTIKAGQPREEQVIMTSQVSLTCLVKGFYIP</td>
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<th>pH52H4-160</th>
<th>SDIAVEWESNGQPPENNYKTTPPLDSDGSSFLYSKLTVDKSRSQGNVF</th>
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</tr>
<tr>
<td>pH52-8.0</td>
<td>SDIAVEWESNGQPPENNYKTTPPLDSDGSSFLYSKLTVDKSRSQGNVF</td>
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<table>
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<tr>
<th>pH52H4-160</th>
<th>CSVMHEALHNHTQKSLSLSPGK</th>
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</tr>
<tr>
<td>pH52-8.0</td>
<td>CSVMHEALHNHTQKSLSLSPGK</td>
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<tr>
<td>450</td>
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FIG. 6B

H52L6-158

10  20  30  
DVQMTQTGSLSASLGDRVTRNCASQDINN
*·****·********·********·********·********·

pH52-9.0

MGWSCIILFLVATATGVHSDIQMTQSPSSLASVGDRVTITCRASQDINN
10  20  30  40  50

pH52-9.0

MGWSCIILFLVATATGVHSDIQMTQSPSSLASVGDRVTITCRASQDINN

40  50  60  70  80

H52L6-158

YLNYWYQQKPNGTVKLIIYTVSLHLGSPRSGSGTIDSYLTISNLQDE
********·······································

pH52-9.0

YLNYWYQQKPGKAPKLIYTVSLHLGSPRSGSGTIDSYLTSSLQDE
60  70  80  90  100

pH52-9.0

YLNYWYQQKPGKAPKLIYTVSLHLGSPRSGSGTIDSYLTISNLQDE

90  100  110  120  130

H52L6-158

DIATYFCQGNTLPPTFGGKTVEIKRRTVAAPSVFIFPSDEQLKSGTAS
*·****·····································

pH52-9.0

DFATYYCQGNTLPPTFGGKTVEIKRRTVAAPSVFIFPSDEQLKSGTAS
110 120 130 140 150

pH52-9.0

DFATYYCQGNTLPPTFGGKTVEIKRRTVAAPSVFIFPSDEQLKSGTAS

140 150 160 170 180

H52L6-158

VVCLNNFYPREAKVQKVGNALQGSNSQESVTEQDSKDTYSLSSTTL
********·····································

pH52-9.0

VVCLNNFYPREAKVQKVGNALQGSNSQESVTEQDSKDTYSLSSTTL
160 170 180 190 200

pH52-9.0

VVCLNNFYPREAKVQKVGNALQGSNSQESVTEQDSKDTYSLSSTTL

190 200 210

H52L6-158

SKADYEKQVACEVTHQGLSSPVTSFNRGEC
********·····································

pH52-9.0

SKADYEKQVACEVTHQGLSSPVTSFNRGEC
210 220 230

pH52-9.0

SKADYEKQVACEVTHQGLSSPVTSFNRGEC

210 220 230
METHOD FOR MAKING HUMANIZED ANTIBODIES

CROSS REFERENCES

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

FIELD OF THE INVENTION

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

BACKGROUND OF THE INVENTION

Naturally occurring antibodies (immunoglobulins) comprise two heavy chains linked together by disulfide bonds and two light chains, one light chain being linked to each of the heavy chains by disulfide bonds. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain (VL) 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. The CDRs in each chain are held in close proximity by the framework regions and, with the CDRs forming 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).

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., Transplantation 41:572–578 (1986)). In a further effort to resolve the antigen binding functions of antibodies and to minimize the use of heterogeneous 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 variable region 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.


For a given antibody a small number of FR residues are anticipated to be important for antigen binding. Firstly for example, certain antibodies bear a conserved 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)).


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 (p185HER2) that is related to and somewhat homologous to the human epidermal growth factor receptor (see Cawsens, 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 p185HER2, specifically inhibits the growth of tumor cell lines overexpressing p185HER2 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 p185HER2 is overexpressed. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. However, this antibody may be immunogenic in humans.

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

It is a further object of this invention to provide methods for the efficient humanization of is 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 p185HER2.

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
variable domain having a CDR and a FR, substituting the 55
body amino acid residue at that site.

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.


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 human antibody 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 this 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 FR residue, determining whether the corresponding consensus FR 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 a 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 this glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

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

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

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

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

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

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

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): DDIOIMTQSPSSLSASV VGVDRITTTSSQDSDDYNTAVAWYQQKPGKAPKLLIYASFLSLEGVPSRFSGSGSDFGTTLTISSLQPEDFAIYYCQQYHTPPFQGKTVEIKRT

SEQ. ID NO. 4 (heavy chain): EVQLVESGGGLVPGGS KLCAASGFNSKTVIWHWWQPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNLSRAEDTAVYFCSRWGDGDFYAMDVWGGQGTLTVSS

**BRIEF DESCRIPTION OF THE DRAWINGS**

**FIG. 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). **FIG. 1B** shows the comparison between the V_{L} 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 1B, the sequences are provided in the accepted numbering scheme.
Interest in technological interest, 5' pH 5.2-9.0 based (Kabat, E. A. et al., 
sequences for the heavy chain of an anti-CD18 antibody. 
sequence. pH 5.2-8.0 residue S55 plus R66 (see Table 3) are shown.
FIG. 4 shows a stereo view of α-carbon tracing for a model of huMAb4D5-8 V_<sub>H</sub> and V_<sub>L</sub>. 
FIG. 5 shows amino acid sequence comparison of V_<sub>H</sub> (top panel) and V_<sub>L</sub> (lower panel) domains of the murine 
anti-CD3 monoclonal Ab UCHT1 (muCD3, Shalaby et al., J. Exp. Med. 175, 217-225 (1992)) with a humanized variant of 
this antibody (huCD3). Also shown are consensus sequences (most commonly occurring residue or pair of residues) of the most abundant human subgroups, namely V_<sub>H</sub> κ I and V_<sub>L</sub> III upon which the humanized sequences are 
based (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_<sub>H</sub> residues A71, T73, A78, S95, Y102 and V_<sub>L</sub> residues Y55 plus R66 (see Table 3) are shown.
FIG. 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 
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_<sub>H</sub> and residue 144A is the first amino acid in the constant heavy chain domain C_<sub>H</sub>.
FIG. 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 
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_<sub>L</sub> and residue 129V is the first amino acid in the light chain constant domain C_<sub>L</sub>.

**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<sub>Hu</sub>. The muMAb4D5 and its uses are described in 
PCT application WO 89/06692 published Jul. 27, 1989. This 
murine antibody was deposited with the ATCC and design­
ated 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 substanc­
tially 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 substanc­
tially 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 immunoglob­
ulin. 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 
ehibit cytotoxic activity, and the class is typically IgG1. 
Where such cytotoxic activity is not desirable, the constant 
domain may be of the IgG2 class. The humanized antibody may 
compass sequences from more than one class or 
isotype, and selecting particular constant domains to opti­
mize 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 mutated 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 prod­
ucts using three dimensional models of the parental and 
humanized sequences. Three dimensional immunoglobulin 
models are commonly available and are familiar to those.
skilled in the art. Computer programs are available which illustrate and display probable three dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen.

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

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

A residue that interacts with a CDR generally is a residue that either affects the conformation of the CDR polypeptide backbone or forms a noncovalent bond with a CDR residue side chain. Conformation-affecting residues ordinarily are those that change the spatial position of any CDR backbone atom (N, Ca, C, O, P) 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_{H}–V_{L} 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_{H} residues 34, 36, 38, 44, 46, 87, 89, 91, 96, and 98 and V_{L} 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 \( V_\gamma \) domain the two cysteine residues are typically at residue numbers 23 and 88, and in the \( V_\delta \) 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 immunoglobulin subunits of a particular species or of many species. A “consensus” sequence, structure, or antibody is understood to encompass a consensus human sequence as described in certain embodiments of this invention, and to refer to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all human immunoglobulins of any particular subclass or subunit structure. This invention provides consensus human structures and consensus structures which consider other species in addition to human.

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

<table>
<thead>
<tr>
<th>Class</th>
<th>Heavy Chain Subclasses</th>
<th>Light Chain Molecular Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>( \gamma_1, \gamma_2, \gamma_3, \gamma_4 )</td>
<td>( \kappa ) or ( \lambda )</td>
</tr>
<tr>
<td>IgA</td>
<td>( \alpha_1, \alpha_2 )</td>
<td>( \kappa ) or ( \lambda )</td>
</tr>
<tr>
<td>IgM</td>
<td>( \mu )</td>
<td>( \kappa ) or ( \lambda )</td>
</tr>
<tr>
<td>IgD</td>
<td>( \delta )</td>
<td>( \kappa ) or ( \lambda )</td>
</tr>
<tr>
<td>IgE</td>
<td>( \epsilon )</td>
<td>( \kappa ) or ( \lambda )</td>
</tr>
</tbody>
</table>

In preferred embodiments of an IgG\( \gamma \) 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 \( V_\gamma \) subgroup I and \( V_\gamma \) group III. In such preferred embodiments, the \( V_\gamma \) consensus domain has the amino acid sequence:

\[
\text{EVQLVESGGGLVPRGSHLRLSCAASGFTFYDYMSTVRQAPGKGLEWAVISENGGTYYADSVKGRFTISADTSKNTAYLQLMNSRAEDTVAVYYCGERDGYAMVWQGGLTHTVSS}
\]

(SEQ. ID NO. 4).

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

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

Identity or homology with respect to a specified amino acid sequence of this invention is defined herein as the percentage of amino acid residues in a candidate sequence that are identical with the specified residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into the specified sequence shall be construed as affecting homology.

All sequence alignments called for in this invention are such maximal homology alignments. While such alignments may be done by hand using conventional methods, a suitable computer program is the “Align 2” program for which protection is being sought from the U.S. Register of Copyrights (Align 2, by Genentech, Inc., application filed Dec. 9, 1991).

“Non-homologous” import antibody residues are those residues which are not identical to an 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:

\[
\text{DIQMTQSPSSLSASVGRVTVTITCRASODVNTAYWQQKPGKAPKLLYYASIFSLGVPFRSGSGGTGDFLTSSLQPEAFTYCVQQHYTTPFGQGTKVEIKRT}
\]

(SEQ. ID NO. 4), which is the light chain variable domain of huMAb4D5; or

\[
\text{EVQLVESGGGLVPRGSHLRLSCAASGFTFYDYMSTVRQAPGKGLEWAVISENGGTYYADSVKGRFTISADTSKNTAYLQLMNSRAEDTVAVYYCGERDGYAMVWQGGLTHTVSS}
\]

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

“Biological property”, as relates for example to anti-p185\(_{HER2}\) binding, any hormonal or hormonal antagonist 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 react-
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 p185ER+.

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 has been substituted by another residue, including predetermined mutations by, e.g., site-directed mutagenesis; derivatives of huMAb4D5 or its fragments as defined above wherein huMAb4D5 or its fragments have been covalently 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 glyco-
sylation site or deletion of any glycosylation site by deletion, insertion or substitution of suitable residues). Such frag-
ments and variants exclude any polypeptide heretofore identi-
fied, including muMAb4D5 or any known polypeptide fragment, which are anticipatory order 35 U.S. 102 as well as polypeptides obvious thereunder under 35 U.S.C. 103.

An “isolated” polypeptide means polypeptide which has been identified and separated and/or recovered from a com-
ponent of its natural environment. Contaminant components of its natural environment are materials which would inter-
ference with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteina-
ceous 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 sequence variants of huMAb4D5 wherein an amino acid residue from at least 5 residues comprising sequences from muMAb4D5 plus residue 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 has been substituted by another residue, including predetermined mutations by, e.g., site-directed mutagenesis; derivatives of huMAb4D5 or its fragments as defined above wherein huMAb4D5 or its fragments have been covalently 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 glyco-
sylation site or deletion of any glycosylation site by deletion, insertion or substitution of suitable residues). Such frag-
ments and variants exclude any polypeptide heretofore identi-
fied, including muMAb4D5 or any known polypeptide fragment, which are anticipatory order 35 U.S. 102 as well as polypeptides obvious thereunder under 35 U.S.C. 103.

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 trans-
lation. Generally, “operably linked” means that the DNA sequences being linked are contiguous and, in the case of a secre-
tory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accom-
plished 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 homolo-
gous 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 design-
ations 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 origi-
nally transformed cell are included. Where distinct design-
ations are intended, it will be clear from the context.

“Oligonucleotides” are short-length, single- or double-
stranded polynucleotides that are chemically synthet-
ized by known methods (such as phosphorotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP 266,032 published May

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 complementa-
tary 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 at 42° C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serumalbumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C.; or (3) employ 50% formamide, 5xSSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5xDenhardt’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 0.2xSSC 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 trans-

ordinarily transformed cell are included. Where distinct desig-
nations are intended, it will be clear from the context.

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

Suitable Methods for Practicing the Invention

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

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

Molecular Modeling

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

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

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

Step 1

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

Table 1. Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure

<table>
<thead>
<tr>
<th>Residue</th>
<th>2FB4</th>
<th>2RHE</th>
<th>2MCP</th>
<th>3FAB</th>
<th>1FBJ</th>
<th>2HFL</th>
<th>1REI</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>IgV domain</td>
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<td>60-66</td>
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<tr>
<td>RMS</td>
<td>0.40</td>
<td>0.60</td>
<td>0.53</td>
<td>0.54</td>
<td>0.48</td>
<td>0.50</td>
<td>0.50</td>
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<td>IgL domain</td>
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<tr>
<td>57-61</td>
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<td>56-60</td>
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<td>68-71</td>
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</tr>
<tr>
<td>RMS</td>
<td>3-8</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

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

Step 3
With the seven structures thus superimposed, for each residue in the template (2FB4) Fab one calculates the distance from the template alpha-carbon atom (Ca) to the analogous Ca atom in each of the other six superimposed structures. This results in a table of Ca-Ca 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 Ca-Ca distances for a given residue position were ±1.0 Å, that position was included in the consensus structure. If for a given position only one Fab crystal structure was >1.0 Å, the position was included but the outlying crystal structure was not included in the next step (for this position only). In general, the seven β-strands were included in the consensus structure while some of the loops connecting the β-strands, e.g., complementarity-determining regions (CDRs), were not included in view of Ca divergence.

Step 4
For each residue which was included in the consensus structure after step 3, the average of the coordinates for individual maincahin 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 published by Kabat, et al. (1985). Standard geometry values are from the AMBER forcefield as implemented in DISCOVER (Biosym Technologies).

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

<table>
<thead>
<tr>
<th>Table II</th>
<th>Average Bond Lengths and Angles for “Average” (Before) and Energy-Minimized Consensus (After 50 Cycles) Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vh (Å)</td>
</tr>
<tr>
<td>N–Cα</td>
<td>1.4506</td>
</tr>
<tr>
<td>N–Cβ</td>
<td>1.282</td>
</tr>
<tr>
<td>Cα–Cβ</td>
<td>1.507</td>
</tr>
<tr>
<td>C–N</td>
<td>1.10</td>
</tr>
<tr>
<td>Cα–C</td>
<td>1.16</td>
</tr>
<tr>
<td>Cβ–C</td>
<td>1.23</td>
</tr>
<tr>
<td>C–N</td>
<td>1.10</td>
</tr>
<tr>
<td>Cα–Cβ</td>
<td>1.11</td>
</tr>
</tbody>
</table>

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 model of any import, human, or humanized Fab can be constructed as follows. Using the amino acid sequence of the particular antibody $V_L$ and $V_H$ domains of interest, a computer graphics program (such as INSIGHT II, 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 not to 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 II (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 Brucoleri 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.

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 $V_L$ 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 (i), 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, 58H, 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 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 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 least at one of the following sites:


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 $V_\gamma-V_\delta$ 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 substitute the amino acids at the specific locations in 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, SOCl₂, or RN₃N=CR=NR, where R and R 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 ½ to ¾ 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 thymi­line (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); Takeya 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 DNA 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 membrane anchoring characteristics, and/or altering the intra-cellular location of the target polypeptide by inserting, deleting, or otherwise affecting any leader sequence of the native target polypeptide.

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

A useful method for identification of certain residues or regions of the target polypeptide that are preferred locations for mutagenesis is called “alanine scanning 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 polyanalnine) 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 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 inframe insertions of single or multiple amino acid residues. Inframe 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 cells. Suitable sequences include STI or Ipp for E. coli, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of the target polypeptide include the fusion to the N- or C-terminus of the target polypeptide of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded on a plasmid for mammalian expression in E. coli. The N-terminal signal sequence of the target polypeptide is 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 on 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 cross-linking. DNA encoding amino acid sequence variants of the target polypeptide is isolated and fused to the signal sequence of the homologous regions of the molecule.

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.

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 give the selected alteration in the target polypeptide DNA.

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

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

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase 1, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for
synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the target polypeptide, and the other strand (the original template) encodes the native, unaltered sequence of the target polypeptide. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as E. coli JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyribodideoxynucleosine (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 mutated. 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 deoxyribonucleotides, 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 preferable for the template sequence of the second primer to be located within 200 nucleotides from that of the first, such that in the end the entire amplification 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 enzyme. If the amino acids are located close together 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 deoxynucleotides triphosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.), and 25 pmole of each oligonucleotide primer, to a final volume of 50μl. The reaction mixture is overlaid with 35 μl mineral oil. The reaction is denatured for 5 minutes at 100°C, placed briefly on ice, and then 1 μl Thermus aquaticus (Taq) DNA polymerase (5 units/μl purchased from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows: 2 min. at 55°C, then 30 sec. at 72°C, then 15 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 another 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, a signal sequence that is complementary to a sequence found in Bacillus species may be suitable. For eukaryotic cells that do not recognize and process the native target polypeptide, a signal sequence is required that is recognized and processed by the eukaryotic signal peptidase.

(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=1: 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 Uralba 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, to which they are operably linked. This amplification technique can be used irrespective of whether the target polypeptide is encoded by DNA sequences native to the source DNA by restriction enzyme digestion and insertions into mammalian expression vectors. Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem., 255: 2073 [1980] or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg., 2: 149 [1968]; and Holland, Biochemistry, 17: 4900 [1978]), such as asenolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucone 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, isocitrycrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also ate advantageously used with yeast promoters.

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

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

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that

(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 generally orientation and position independent having been found 5' (Laaimns et al., *Proc. Natl. Acad. Sci. USA*, 83: 993 [1981]) and 3' (Usky et al., *J. Mol. Cell Bio.*, 3: 1108 [1983]) to the transcription initiation unit, within an intron (Banerji et al., *Cell*, 33: 729 [1983]) as well as within the coding sequence itself (Osborne et al., *J. Mol. Cell Bio.*, 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, \( \alpha \)-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 sequences 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 ligated in the form desired to generate the plasmids required.

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

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

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the target polypeptide 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 pSV16B.

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 *S. subtilis*, Pseudomonas species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescens*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, in vitro methods of cloning, e.g. PCR or other nucleic acid polymerase reactions for activation of the target polypeptide.

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 bacterial vector Agrobacterium tumefaciens, which has been previously manipulated to contain the target polypeptide DNA. During incubation of the plant cell culture with A. tumefaciens, the DNA encoding target polypeptide is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the target polypeptide DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al., J. Mol. Appl. Gen., 1: 561 (1982).

In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published Jun. 21, 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CVI line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line 293 or 293 cells subcloned for growth in suspension culture, Gra-4575; human liver cells (Hep G2, ATCC CRL 10) or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the target polypeptide currently in use in the field.

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, CaPO4 and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., supra, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers.

Infection with Agrobacterium tumefaciens is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23: 315 (1983) and WO 89/05859 published Jun. 29, 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30–16.37 of Sambrook et al., supra, is preferred. General aspects of mammalian cell host system transformations have been reviewed by Axel in U.S. Pat. No. 4,390,216 issued Aug. 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130: 946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

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. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. Re. 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamicin™ 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 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 32P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a sample, fixing cells to a surface, such as glass, plastic, or a membrane, and staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Various labels may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the presence of antibody bound to the DNA 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.

Alternatively, antibody 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 immunogenic site, such as a phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibodies 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 bromomethylformamide, α-bromo-β-(5-imidozoyl)propionic acid, chloroaacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl2-pyridyldisulfide, p-chloromercurobenzoate, 2-chloromercuro-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.

Lysyl 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 lysyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidocetates such as methyl picolinimidate; pyridoxal phosphate; pyridoxal;
chloroborohydride; trinitrobenzenesulfonic acid; O-methylsourea, 2,4-pentaneone; 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 pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in so introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetraniromethane. Most commonly, N-acetylglucosimide and tetraniromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using 125I or 131I 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 carboxidimides (R-N=C=O), 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, glutaeralddehyde, N-hydroxysuccimidine esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including dianiscuimidinyl esters such as 3,3'-dithiodisuccinimidyl propionate, and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanoen bromide-activated carbohydrates and the reactive substrates are described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,459; 4,229,537; and 4,257,306.

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-acetylgalactosamine or N-acetylgalactosaminate), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin et al. (Arch. Biochem. Biophys., 259:52 [1987]) and by Edge et al. (Anat. Biochem., 116:131 [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 of polypeptides is typically either N-linked or O-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tri-peptide sequences asparagine-X-resine 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 glycosylation sites include tyrosyl residues or hydroxyamino acid, almost 5-hydroxyproline or 5-hydroxlysine 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, the target polypeptide amino acid sequences are not altered but are modified through changes at the DNA level, particularly by mutating the DNA encoding the target polypeptide at preselected bases such that sequences that require translation 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 type of chemical or enzymatic modification of the target polypeptide comprises linking the target polypeptide to various nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site. 0-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.
manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. The target polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxyethylcellulose or gelatin-microcapsules and poly-[methylmethacrylate]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, 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 radio receptor 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 recombiant 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 upon the particular antigen or antibody, and proteins that bind to the analyte are denominated binding partners, irrespective of their status 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, $^{252}$P, $^{3}$H, and $^{15}$I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalalizedinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucamylase, 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, biotinavidin, 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 diamines, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. No. 3,940,475 (fluorimetry) and U.S. Pat. No. 3,645,090 (enzymes); Hunter et al., Nature, 144: 945 (1962); David et al., Biochemistry, 13: 1014–1021 (1974); Pain et al., J. Immunol. Methods, 40: 219–230 (1981); and Nygren, J. Histochem. and Cytochem., 30: 407–412 (1982).

Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase.


Immunobilization 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 immobilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. Pat. No. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by immobilizing 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 immobilized before or after the competition and then the tracer and analyte bond to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preimmobilized) or by centrifuging (where the binding partner was added 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-enzyme binds to the analyte the presence of the anti-enzyme modifies the enzyme activity. In this case, the antigen or its immunologically active fragment(s) 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 proceeds 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 the analyte, thereby allowing anti-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 may be used hereafter for the determination of these analytes are included within the scope hereof, including the bioassays described above.

Immunotoxins

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

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

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal, plant or animal origin, or an enzymatically active fragment of such a toxin. Enzymatically active toxins and fragment thereof are used, for example, to cleave DNA or protein, and for example to cleave DNA to block DNA replication, and to cleave proteins to block cell protein synthesis. Other biological effects may be achieved using immunotoxins.

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 Fe region of the antibody molecule.
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 (Vaname 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 antiidiotypic antibodies (Nepom et al., *Proc. Natl. Acad. Sci. USA* 81:2864 (1985); Koprowski et al., *Proc. Natl. Acad. Sci. USA* 81:216 (1984)) relating to such antigens could be used to induce an active immune response in human patients. Such a response includes the formation of antibodies capable of activating human complement and mediating ADCC and by such mechanisms cause infected cell destruction.

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

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

Deposit of Materials

As described above, cultures of the muMAB4D5 have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Va., USA (ATCC).

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

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

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

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the constructs deposited, since the deposited embodiments are intended to illustrate only certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention.

EXAMPLES

Example 1

Humanization of muMAb4D5

Here we report the chimerization of muMAb4D5 (chMAb4D5) and the rapid and simultaneous humanization of heavy (VH) and light (VL) 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 p185HER and anti-proliferative activity against p185HER overexpressing carcinoma cells.

Materials and Methods

Cloning of Variable Region Genes. The muMAb4D5 VH and VL 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 VH and VL 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)) in combination with restriction sites for directional cloning shown by underlining and listed after the sequences: VH sense, 5'-TCC

GATATCCAGCTGACCGATGCCCTCCA-3' (SEQ. ID NO. 7), EcoRV, Y, anti-sense, 5'-GGTTGAATTCAGCTT

GTTACCHSDCCGAA-3' (SEQ. ID NO. 8), Asp718; VH sense, 5'-AGGTSMARCTGCAGSAGTCWG-3' (SEQ. ID NO. 9), Psil and VH ant-sense, 5'-TGAAGGAGAC

GTGACCGTGTTCCTGGCCCAG-3' (SEQ. ID NO. 10), BstEII; where H=A or C or T, S=C or G, D=A or G or T, M=A or C, R=A or G and W=A or T. The PCR products were cloned into pUC119 (Vieira, J. & Messing, J., Methods Enzymol. 153:3–11 (1987)) and five clones for each variable domain sequenced by the dideoxy method (Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74:5463–5467 (1977)).

Molecular Modelling. Models for muMAb4D5 VH and VL domains were constructed separately from consensus coordinates based upon seven Fab structures from the Brookhaven protein data bank (entries 1F8B, 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 VH and VL domains and additional structures were then superimposed upon this structure using their main chain atom coordinates (INSIGHT program, Biosym Technologies). It is understood that the distance from the Cα of each of the superimposed structures was calculated for each residue position. If all (or nearly all) Cα—Cα distances for a given residue were ≤1 Å, then that position was included in the consensus structure. In most cases the β-sheet framework residues satisfied this criteria whereas the CDR loops did not. For each of these selected residues the average coordinates for individual N, Cα, 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 VH and VL 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 VH and VL 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 VH κ subgroup I and VH κ 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 defined by structural variability (Chothia, C. & Lesk, A. M., J. Mol. Biol. 196:901–917 (1987)):

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 Centricron-100, Amicon), sterile-filtered (Millex-GV, Millipore) and stored at 4°C. The concentration of antibody was determined by both total immunoglobulin and antigen binding ELISAs. The standard used was huMAb4D5-5, whose concentration had previously 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 MA b4D5 concentrations.

Affinity Measurements. The antigen binding affinity of MAb4D5 variants was determined using a secreted form of the p185HER ECD prepared as described in Fendly, B. M. et al., J. Biol. Resp. Mod. 9:449–455 (1990). Briefly, antibody and p185HER ECD were incubated in solution until equilibrium was found to be reached. The concentration of free antibody was then determined by ELISA using immobilized p185HER ECD and used to calculate affinity (Kd) according to Friguet et al. (Friguet, B. et al., J. Immunol. Methods 77:305–319 (1985)).

Results

HUMANIZATION OF MAb4D5. The muMAb4D5 VH and VL gene segments were first cloned by PCR and sequenced (FIG. 1). The variable genes were then simultaneously humanized by gene conversion mutagenesis using pre-assembled oligonucleotides (FIG. 2). A 311-mer 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 MgCl2 as above. Heteroduplex DNA was constructed by extending the primers with T7 DNA polymerase and transformed into E. coli BM17–18 mutl as previously described (Carter, P., in Mutagenesis: A Practical Approach, Chapter 1 (IRL Press, Oxford, UK 1991)). The resultant phagemid DNA pool was enriched first for huVH by restriction purification using XhoI and then for huVL by restriction selection using Stul 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 huVH and huVL 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 VH and VH gene segments in the transient expression vectors described above were then precisely replaced with their humanized versions.

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otide containing 39 mismatches to the template directed 24 simultaneous amino acid changes required to humanize muMAb4D5 V\textsubscript{H}. Humanization of muMAb4D5 V\textsubscript{H} required 32 amino acid changes which were installed with a 361-mer oligonucleotide containing 39 mismatches to the template directed 24 plates allowed 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\textsubscript{HER2} ECD. Successive harvests of five 10 cm plates allowed 200 μg to 300 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\textsubscript{r} of ~130 kDa. Electrophoresis under reducing conditions gave 2 bands consistent with the expected M\textsubscript{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 V\textsubscript{H} 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\textsubscript{H} residues 71, 73, 78, 93 plus 102 and V\textsubscript{L} residues 55 plus 66 identified by our molecular modeling. V\textsubscript{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\textsubscript{H}-CDR2. Amino acid sequence differences between huMAb4D5 variant molecules are shown in Table 3 together with their p185\textsubscript{HER2} ECD binding affinity and maximal anti-proliferative activities against SK-BR-3 cells. Very similar K\textsubscript{s} values were obtained for binding of huMAb4D5 variants to either SK-BR-3 cells or to p185\textsubscript{HER2} ECD (Table 3). However, K\textsubscript{s} estimates derived from binding of huMAb4D5 variants to p185\textsubscript{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\textsubscript{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 huMAb4D5 variant, created by simply installing the muMAb4D5 CDRs into the consensi human sequences. huMAb4D5-1 binds the p185\textsubscript{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\textsubscript{HER2} overexpressing SK-BR-3 cells is not simply correlated with their binding affinity for the p185\textsubscript{HER2} ECD. For example, installation of three murine residues into the V\textsubscript{H} domain of huMAb4D5-2 (D73F, 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\textsubscript{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\textsubscript{HER2} ECD on replacement of R71 in huMAb4D5-1 with the corresponding murine residue, alanine (huMAb4D5-2). In contrast, replacing V\textsubscript{H} L78 in huMAb4D5-4 with the murine residue, alanine (huMAb4D5-5), does not significantly change the affinity for the p185\textsubscript{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\textsubscript{HER2}.

V\textsubscript{H} 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 huMAb4D5 κ light chain. The side chain of residue 66 is likely to affect the conformation of V\textsubscript{L}-CDR1 and V\textsubscript{H}-CDR2 and the hairpin turn at 68–69 (FIG. 4). Consistent with the importance of this residue, the mutation V\textsubscript{L} G66R increases the affinity for the p185\textsubscript{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\textsubscript{H} residue 55 may either stabilize the conformation of V\textsubscript{H}-CDR3 or provide an interaction at the V\textsubscript{H}–V\textsubscript{L} interface. The latter function may be dependent upon the presence of V\textsubscript{H} Y102. In the context of huMAb4D5-5 the mutations V\textsubscript{L} E55Y (huMAb4D5-6) and V\textsubscript{H} Y102Y (huMAb4D5-7) individually increase the affinity for p185\textsubscript{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\textsubscript{L} Y55 and V\textsubscript{H} Y102.

Secondary Immune Function of huMAb4D5-8.

MuMAb4D5 inhibits the growth of human breast tumor cells which overexpress p185\textsubscript{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\textsubscript{d}=0.1 μM) and its human IgG\textsubscript{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\textsubscript{HER2} and its human IgG\textsubscript{1} subtype. The results demonstrate that: (1) huMAb4D5 has a greatly enhanced ability to carry out ADCC as compared to its murine parent; and (2) that this activity may be selective for cell types which overexpress p185\textsubscript{HER2}.

Discussion

MuMAb4D5 is potentially useful for human therapy since it is cystostatic towards human breast and ovarian tumor lines overexpressing the HER2-encoded p185\textsubscript{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 cystostatic rather than solely cystostatic in effect. Humanization of muMAb4D5 should accomplish these goals. We have identified 5 different huMAb4D5 variants which bind tightly to p185\textsubscript{HER2} ECD (K\textsubscript{s}≤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\textsubscript{HER2} in the presence of human effector cells (Table 4) as anticipated for a human γ1 isotype (Brcüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987); Riechmann, L. et al., *Nature* 332:323–327 (1988)).
Rapid humanization of huMAb4D5 was facilitated by the gene conversion mutagenesis strategy developed here using long preassembled oligonucleotides. This method requires less than half the amount of synthetic DNA as does total gene synthesis and does not require convenient restriction sites in the target DNA. Our method appears to be simpler and more reliable than a variant protocol recently reported (Rostapshov, V. M. et al., FEBS Lett. 249: 379–382 (1989)). Transient expression of huMAb4D5 in human embryonic kidney 293 cells permitted the isolation of a few hundred micrograms of huMAb4D5 variants for rapid characterization by growth inhibition and antigen binding affinity assays. Furthermore, different combinations of light and heavy chain were readily tested by co-transfection of corresponding cDNA expression vectors.

The crucial role of molecular modeling in the humanization of muMAb4D5 is illustrated by the designed variant huMAb4D5-8 which binds the p185HER2 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 p185HER2 ECD. For example the huMAb4D5-8 variant binds p185HER2 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 p185HER2 allows for the evolution of a straightforward clinic approach to those human cancers characterized by overexpression of the HER2 protooncogene.

### Table 3

| p185HER2 ECD binding affinity and anti-proliferative activities of MAb4D5 variants |
|---------------------------------|----------------|----------------|----------------|----------------|
| V<sub>0</sub> Residue*          | V<sub>0</sub> Relative |
|---------------------------------|----------------|----------------|----------------|----------------|
| MAb4D5 cell variant proliferation<sup>1</sup> | 71 | 73 | 78 | 93 | 102 | 55 | 56 | K<sub>a</sub> mN 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 | 106 |
| huMAb4D5-4                      | Ala | Thr | L | Ser | V | E | Arg | 0.32 | 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 | E | Arg | 0.10 | 54 |
| muMAb4D5                        | Ala | Thr | Ala | Ser | Tyr | E | Arg | 0.30 | 37 |

<sup>1</sup>Human and murine residues are shown in one letter and three letter amino acid code respectively.

<sup>2</sup>Proliferation of SK-BR-3 cells incubated for 96 hr with MAb4D5 variants shown as a percentage of control.

<sup>3</sup>Selection of antibody dependent tumor cell cytotoxicity mediated by huMAb4D5-8.

### Table 4

<table>
<thead>
<tr>
<th>Effector: Target</th>
<th>WI-38&lt;sup&gt;*&lt;/sup&gt;</th>
<th>SK-BR-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio*</td>
<td>muMAb4D5</td>
<td>huMAb4D5-8</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------</td>
<td>----------</td>
</tr>
<tr>
<td>25:1</td>
<td>&lt;1.0</td>
<td>9.3</td>
</tr>
<tr>
<td>12.5:1</td>
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<td>8.9</td>
</tr>
<tr>
<td>3.13:1</td>
<td>&lt;1.0</td>
<td>8.5</td>
</tr>
<tr>
<td>B</td>
<td>25:1</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>12.5:1</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>6.25:1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>3.13:1</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

<sup>*</sup>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 p185HER2 (64 pg p185HER2 per pg cell protein) and SK-BR-3 expresses a high level of p185HER2 (64 pg p185HER2 per pg cell protein), as determined by ELISA (Fendly et al., J. Biol. Resp. Mod. 9:449-455 (1989)). 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 51Cr release. Estimated standard error in these quadruplicate determinations was ±10%.

<sup>1</sup>Monoclonal antibody concentrations used were 0.1 µg/ml (A) and 0.1 µg/ml (B).
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 essential to the claimed invention, and that steps may be taken in different order.

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 being considered 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, 35L, 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 effect 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 to the Cho et al., Nature 342:877 (1989), and residues appearing in italic were altered during humanization by the 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


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

ii. Variable heavy domain:


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 Fab'_{2} Fragment

This example demonstrates the construction of a humanized bispecific antibody (BsF(ab')_{2}) by separate E. coli expression of each Fab' arm followed by directed chemical coupling in vitro. BsF(ab')_{2} (anti-CD3/anti-p185\textsubscript{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 p185HER2 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-p185HER2 arm of BsF(ab')_2 vl. In contrast, BsF(ab')_2 vl binds to T cells via its anti-CD3 arm much less efficiently than does the chimeric BsF(ab')_2 vl, which contains the variable domains of the murine parent anti-CD3 antibody. Here we have constructed additional BsF(ab')_2 fragments containing variant anti-CD3 arms with selected murine residues restored in an attempt to improve antibody binding to T cells. One such variant, Ss F(ab')_2 v9, was created by replacing six residues in the second hypervariable loop of the anti-CD3 heavy chain variable domain of BsF(ab')_2 vl with their counterparts from the murine parent anti-CD3 antibody. BsF(ab')_2 vl binds to T cells (Jurkat) much more efficiently than does BsF(ab')_2 v9 and almost as efficiently as the chimeric BsF(ab')_2 vl. This improvement in the efficiency of T cell binding of the humanized BsF(ab')_2 vl is an important step in its development as a potential therapeutic agent for the treatment of p185HER2-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 (1990); Fanger, M. W. et al., J. Exp. Med. 175: 217–225 (1992)). 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 fragment (anti-FLAG chelate/anti-carcinoembryonic antigen) allowed clinical imaging of colorectal carcinoma (reviewed by Songsivilai, W. et al., Biochim. Biophys. Acta 1040: 11–23 (1991)). The expression plasmid, pAK19, for the co-secretion 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 92 (1992a) and Carter, P., et al., Biol. Technology 10: 163–167 (1992b)) of the murine monoclonal Ab 4D5 which is directed against the p185HER2 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 minimally 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 (Beverly, P. C. L. and Callard, R. E., Eur. J. Immunol. 11: 329–334 (1981)) into the humanized anti-p185HER2 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 p185HER2 and to human peripheral blood mononuclear cells carrying CD3. In addition, BsF(ab')_2 v1 enhanced the cytotoxic effects of activated human CTL 4-fold against SK-SR-3 tumor cells overexpressing p185HER2. The example describes 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) domain sequences 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 number assigned 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:

**Example 1:**

- **HX11, 5′-TGA(T)ATATCC1cttACACGCT-3′** (SEQ.ID. NO. 13) V_H K75S: N76S, v8;
- **HX12, 5′-TGA(T)ATAATCC1cAtACACGCT-3′** (SEQ.ID. NO. 12) V_H N76S, v7;
- **HX13, 5′-TGA(T)ATAATCC1cAtACACGCT-3′** (SEQ.ID. NO. 13) V_H K75S:N76S, v8;
- **HX14, 5′-CTTATAAAGGGTTTCCAcACCTATAcCC aG aAt TC1CAAGGatCGT7TTCACgATAgC-3′** (SEQ.ID. NO. 14) V_H T175S:G60N:D616Q:S62K:V63F:O65S, v9;
- **LX6, 5′-CTACATCTCGCGTGcTarCTCGGAGTCTCC-3′** (SEQ.ID. NO. 15) v_H, E129H, v11;

Oligonucleotides HX11, HX12 and HX13 each remove a unique restriction 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 dideoxy-nucleotide sequencing (Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74: 5463–5467 (1977)).

*E. coli* Expression of Fab' Fragments

The expression plasmid, pAg19, for the co-secretion of light chain and heavy chain Fab' fragment of the most preferred humanized anti-p185HER2 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 ohsA promoter. Genes encoding humanized V<sub>H</sub> and V<sub>L</sub> 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<sub>1</sub>, C<sub>1</sub>, and IgG1C<sub>2</sub>1 constant domain genes, respectively. The C<sub>2</sub> gene is immediately followed by a sequence encoding the hinge sequence CysAlaAla and followed by a bacteriophage λ l<sub>1</sub> 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-p185HER<sub>2</sub> V<sub>H</sub> and V<sub>L</sub> 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-p185HER<sub>2</sub> Fab' fragment was secreted from E. coli K12 strain 25S2 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<sub>600</sub> and the titer of soluble and functional anti-p185HER<sub>2</sub> 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 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')<sub>2</sub> 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). Thiocysteine linked BsF(ab')<sub>2</sub> fragments (anti-p185HER<sub>2</sub>/anti-CD3) were constructed by the procedure of Glennie et al. supra with the following modifications. Anti-p185HER<sub>2</sub> 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-phenylenediamine (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 5.5) (coupling buffer) using concentrators (Amicon). The total concentration of Fab' variants was estimated from the measured absorbance at 280 nm (HuMab4D5-8 Fab' ε<sub>280</sub> = 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-p185HER<sub>2</sub> Fab'-mal (assuming quantitative reaction of Fab'-SH with o-PDM) and each anti-CD3 Fab'-SH variant were coupled to a common combination 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 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')<sub>2</sub> was isolated from the coupling reaction by size exclusion chromatography (2.5 cm x 100 cm) in the presence of PBS. The BsF(ab')<sub>2</sub> samples were then filtered through a 0.2 mm filter, flash frozen in liquid nitrogen and stored at ~70° C. Flow Cytometric Analysis of F(ab')<sub>2</sub>Binding to Jurkat Cells

The Jurkat human acute T cell leukemia cell line was purchased from the American Type Culture Collection (Manassas Va.) (ATCC TIB 152) and grown as recommended by the ATCC. Aliquots of 10° Jurkat cells were incubated with appropriate concentrations of BsF(ab')<sub>2</sub>, (anti-p185HER<sub>2</sub>/anti-CD3 variant) or control mono-specific anti-p185HER<sub>2</sub>F(ab')<sub>2</sub> 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')<sub>2</sub> (Organon Teknika, West Chester, Pa.) for 45 min at 4° C. Cells were washed and analyzed on a FACSScan® (Becton Dickinson and Co., Mountain View, Calif.). Cells (8x10<sup>6</sup>) 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<sub>H</sub> and at 37 out of 122 positions within V<sub>L</sub> (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<sub>H</sub> CDR2 of anti-CD3 v1 were replaced en bloc with their murine counterparts to give anti-CD3 v9: T57S6A60N:D61Q:S62K:V63F:G65D (SEQ 1D NO:20). Similarly, the human residue E55 in V<sub>L</sub> CDR2 of anti-CD3 v1, was replaced with histidine from the murine anti-CD3 antibody to generate anti-CD3 v11. In addition, V<sub>H</sub> 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<sub>L</sub> residues 75 and 76 are located in a loop close to V<sub>H</sub> 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')<sub>2</sub> Fragments

Soluble and functional anti-p185HER<sub>2</sub> and anti-CD3 Fab' fragments were recovered directly from corresponding E. coli fermentation pastes with the single hinge cysteine predominately 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). Thiocysteine-linked BsF(ab')<sub>2</sub> 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-p185HER<sub>2</sub> variant, HuMab4D5-8 (Carter et al., 1992a, supra) and the other either a chimeric or humanized variant of the anti-CD3 antibody. Anti-p185HER<sub>2</sub> 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')<sub>2</sub> was then purified away from unreacted Fab' by size exclusion chromatography as shown for a representative preparation (BsF(ab')<sub>2</sub>; v8) in data not shown. The F(ab')<sub>2</sub> fragment represents ~54% of the total amount of antibody fragments (by mass) as judged by integration of the chromatography peaks.

SDS-PAGE analysis of this BsF(ab')<sub>2</sub>v8 preparation under non-reducing conditions gave one major band with the expected mobility (M, ~96 kDa) as well as several very minor species.
bands (data not shown). Amino-terminal sequence analysis of the major band after electroblotting on to polyvinylidene difluoride 76 are located in a loop close to $V_H$ CDR1 and CDR2 and therefore might membrane Matsudaira, P., J. Biol. Chem. 262: 10035–10038 (1987) gave the expected mixed sequence from a stoichiometric 1:1 mixture of light and heavy chains ($V_L/N_p$: D/E, I/V, Q/D, M/L, T/V, D/E, S/S) expected for $B_{sF'(ab')}$. 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-p18$^{HER_2}$ and anti-CD3 antigen specificities. (Shalaby et al., supra). The level of contamination of the $B_{sF'(ab')_2}$ with monospecific $F(ab')_2$ is likely to be very since low mouse coupling reactions with either anti-p18$^{HER_2}$ w Fab'-mal or anti-CD3 Fab'-SH alone did not yield detectable quantities of $F(ab')_2$. Furthermore the coupling reaction was subjected to a mild reduction step followed by alklylation 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 $B_{sF'(ab')_2}$ to Jurkat Cells

Binding of $B_{sF'(ab')_2}$, containing different anti-CD3 variants to Jurkat cells (human acute T cell leukemia) was investigated by flow cytometry (data not shown). $B_{sF'(ab')_2}$v9 binds much more efficiently to Jurkat cells than does our starting molecule, $B_{sF'(ab')_2}$v1, and almost as efficiently as the chimeric $B_{sF'(ab')_2}$. Installation of additional murine residues into anti-CD3 v9 to create v10 ($V_H/K75S:N76S$) and v12 ($V_L/K75S:N76S$ plus $V_E/E55H$) did not further improve binding of corresponding $B_{sF'(ab')_2}$ to Jurkat cells. Nor did recruitment of these murine residues into anti-CD3 v1 improve Jurkat binding: $V_H/K75S$ (v6), $V_L/N76S$ (v7), $V_H/K75S:N76S$ (v8), $V_E/E55H$ (v11) (not shown). $B_{sF'(ab')_2}$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-p18$^{HER_2}$ $F(ab')_2$ 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-p18$^{HER_2}$ (Carter et al., 1992a, supra) and anti-CD3 arms (Shalaby et al., supra) of the $B_{sF'(ab')_2}$ 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-p18$^{HER_2}$ antibody where one out of eight humanized variants ($HuMAb4D5-8$, IgG1) was identified that bound the p18$^{HER_2}$ antigen ~3-fold more tightly than the parental murine antibody (Carter et al., 1992a, supra). $HuMAb4D5-8$ contains a total of five murine FR residues and nine murine CDR residues, including $V_L/K75S$ and CDR residues, were discarded in favor of human counterparts. In contrast, $B_{sF'(ab')_2}$v1 containing the most potent humanized anti-CD3 variant 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 $B_{sF'(ab')_2}$.

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:A60D:E61Q: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). $B_{sF'(ab')_2}$v9 binds to SK-BR-3 breast tumor cells with equal efficiency to $B_{sF'(ab')_2}$v1 and chimeric $B_{sF'(ab')_2}$ as anticipated since the anti-p18$^{HER_2}$ arm is identical in all of these molecules (Shalaby et al., supra, not shown).

Our novel approach to the construction of $B_{sF'(ab')_2}$ 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 $B_{sF'(ab')_2}$, 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 $B_{sF'(ab')_2}$ preparation such as intra-hinge disulfide formation and contamination with intact parent antibody whilst greatly diminishes others, eg. formation of Fab'(ab')3 fragments.

$B_{sF'(ab')_2}$ 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 Fab'v1 may be more stable than disulfide-linked Fab'v1 in vivo (Glennie et al., supra). This hypothesis is supported by our preliminary pharmacokinetic experiments in normal mice which suggest that thioether-linked $B_{sF'(ab')_2}$v1 has a 3-fold longer plasma residence time than $B_{sF'(ab')_2}$v1 linked by a single disulfide bond. Disulfide and thioether-linked chimeric $B_{sF'(ab')_2}$ 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
Example 4

Humanization of an anti-CD18 Antibody

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

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu 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 Gln Leu 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 Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75
Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Cys Gln Gln
80 85 90
His Tyr Thr Thr Pro Pro Thr Phe Gly 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 Gly Gly Gly Gly Leu Val Gln Gly Val Gln
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 Lys 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 1 00 1 05
Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
  110 115 120

(2) INFORMATION FOR SEQ ID NO:3:

   (i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 109 amino acids
   (B) TYPE: Amino Acid
   (D) TOPOLOGY: Linear

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

   Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
     1   5   10   15
   Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Ser
     20  25   30
   Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
     35  40   45
   Leu Leu Ile Tyr Ala Ala Ser Ser Leu Gly 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 Gly Asp Phe Ala Thr Tyr Cys Gln Gln
     80  85   90
   Tyr Asn Ser Leu Tyr Thr Phe Gly Gln Gly Thr Val Gly
     95 1 00 1 05
   Ile Lys Arg Thr
    109

(2) INFORMATION FOR SEQ ID NO:4:

   (i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 120 amino acids
   (B) TYPE: Amino Acid
   (D) TOPOLOGY: Linear

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

   Glu Val Gln Leu Val Glu Ser Gly Gly Gln Leu Val Gln Pro Gly
     1   5   10   15
   Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
     20  25   30
   Asp Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
     35  40   45
   Glu Trp Val Ala Val Ile Ser Glu Asn Gly Ser Asp Thr Tyr Tyr
     50  55   60
   Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser
     65  70   75
   Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
     80  85   90
   Thr Ala Val Tyr Tyr Cys Ala Arg Asp Arg Gly Gln Ala Val Ser
     95 1 00 1 05
   Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
    110 115 120
(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 Phe Met Ser Thr Ser Val
1  5  10  15
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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 Ala Val Tyr Cys Gln Gln
80 85 90
His Tyr Thr Thr Pro Pro Thr Phe Gly Gly Thr Lys Leu Glu
95 100 105
Ile Lys Arg Ala
109

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1  5  10  15
Ala Ser Leu Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
20 25 30
Asp Thr Tyr Ile His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu
35 40 45
Glu Trp Ile Gly Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
50 55 60
Asp Pro Lys Phe Gln Asp Lys Ala Thr Ile Thr Ala Asp Thr Ser
65 70 75
Ser Asn Thr Ala Tyr Leu Gln Val Ser Arg Leu Thr Ser Glu Asp
80 85 90
Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
95 100 105
Ala Met Asp Tyr Trp Gly Gln Gly Ala Ser Val Thr Val Ser Ser
110 115 120

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDNESS: Single
(D) TOPOLOGY: Linear

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

TCCGATATCC AGCTGACCCA GTCTCCA

Val 15
Asn 30
Lys 45
Asp 60
Ile 75
Gln 90
Glu 105
Gly 120
(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
GTTGATCTC CAGCTTGGTA CCHSCDCCGA A

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
AGGSMARCT GCAGSAGTCW GG

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAG

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
GTAGATAAAT CCTCTTCTAC AGCCTATCTG CAAATG

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
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(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 68 base pairs
   (B) TYPE: Nucleic Acid
   (C) STRANDEDNESS: Single
   (D) TOPOLOGY: Linear

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

CTTATAAAGG TGTTTCCACC TATAACCAGA AATTCAAGGA TCGTTTCACG
ATATCCGTAG ATAAATCC 68

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 30 base pairs
   (B) TYPE: Nucleic Acid
   (C) STRANDEDNESS: Single
   (D) TOPOLOGY: Linear

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

CTATACCTCC CGTCTGCATT CTGGAGTCCC

(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:

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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 Glu Asp Gly Thr Val Lys 35 40 45
Leu Leu Ile 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
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Asn Tyr Leu Asn Trp Tyr Gln Glu Lys Pro Gly Lys Ala Pro Lys
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(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:

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Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
25   25     30     35
Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35   40     45     50
Leu Leu Ile Tyr Ala Ala Ser Ser Leu Ser Gly Val Pro Ser
50   55     60     65
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
70   75     80     85
Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
90   95     100   105
Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
107  107    112    117

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1    5      10     15
Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr
20   25     30     35
Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu
35   40     45     50
Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
55   60     65     70
Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys Ser
85   90     95     100
Ser Ser Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp
105  110    115    120
(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 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 Ala Gly Thr Thr Val Ser Ser
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 Ala
 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 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 Asp 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 Leu Gly Thr Glu 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 Gly 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 Tyr Ser Lys Leu Thr Val Asp
410 415 420

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

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

Ser Pro Gly Lys
454

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 469 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
1 5 10 15

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

Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly
35 40 45

Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met Arg Glu Ala Pro
50 55 60

Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly
65 70 75

Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser
80 85 90

Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu
95 100 105

Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly
110 115 120

Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gin
125 130 135

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

Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
155 160 165

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
170 175 180

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr
185 190 195

Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser
200 205 210
Val Val Thr Val Thr Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr
215 220 225
Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr
230 235 240
Val Glu Arg Lys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
245 250 255
Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
260 265 270
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
275 280 285
Val Asp Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr
290 295 300
Val Asp Gly Met Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
305 310 315
Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu Thr Val
320 325 330
Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
335 340 345
Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
350 355 360
Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
365 370 375
Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
380 385 390
Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
395 400 405
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu
410 415 420
Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
425 430 435
Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
440 445 450
His Glu Ala Leu His His Tyr Thr Gln Lys Ser Ser Leu Ser Leu
455 460 465
Ser Pro Gly Lys
469

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 214 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

Asp Val Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
1  5  10  15
Gly Asp Arg Val Thr Ile Asn Cys Arg Ala Ser Gln Asp Ile Asn
20  25  30
Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asn Gly Thr Val Lys
35  40  45
Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
50  55  60
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
65  70  75
Ser Asn Leu Asp Gin Glu Asp Ile Ala Thr Tyr Phe Cys Gin Glu 80 85 90
Gly Asn Thr Leu Pro Pro Thr Phe Gly Gly Gly Thr Lys Val Glu 95 1 100 1 105
Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro 110 115 120
Ser Asp Glu Gin 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 Gin Trp Lys Val 140 145 150
Asp Asn Ala Leu Gln Ser Gly Asn Ser Gin Glu Ser Val Thr Glu 155 160 165
Gln Ser Ser Lys Asp Ser Thr Ser Tyr Ser Leu Ser Ser Thr Leu Thr 170 175 180
Leu Ser Lys Ala Asp Tyr Glu Lys His Tyr Ala Cys Glu 185 190 195
Val Thr His Gin 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 Gin Met Thr Gin 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 Thr Leu Asn Trp Tyr Gin Gin 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 Thr Asp Tyr 80 85 90
Thr Leu Thr Ile Ser Ser Leu Gin Pro Glu Asp Ala Thr Tyr 95 100 1 105
Tyr Cys Gin Gin Gly Asn Thr Leu Pro Pro Thr Phe Gly Gin Glu 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 Gin 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 Gin Ser Gly Asn Ser Gin Glu 170 175 180
Ser Val Thr Glu Gin Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser 185 190 195
We claim:

1. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, and 92H, utilizing the numbering system set forth in Kabat.

8. The humanized variable domain of claim 1 wherein the residue at site 44L has been substituted.

9. The humanized variable domain of claim 1 wherein the residue at site 58L has been substituted.

10. The humanized variable domain of claim 1 wherein the residue at site 62L has been substituted.

11. The humanized variable domain of claim 1 wherein the residue at site 65L has been substituted.

12. The humanized variable domain of claim 1 wherein the residue at site 66L has been substituted.

13. The humanized variable domain of claim 1 wherein the residue at site 67L has been substituted.

14. The humanized variable domain of claim 1 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

15. The humanized variable domain of claim 1 wherein the residue at site 68L has been substituted.

16. The humanized variable domain of claim 1 wherein the residue at site 69L has been substituted.

17. The humanized variable domain of claim 1 wherein the residue at site 73L has been substituted.

18. The humanized variable domain of claim 1 wherein the residue at site 85L has been substituted.

19. The humanized variable domain of claim 1 wherein the residue at site 98L has been substituted.

20. The humanized variable domain of claim 1 wherein the residue at site 2H has been substituted.
21. The humanized variable domain of claim 1 wherein the residue at site 36H has been substituted.

22. The humanized variable domain of claim 1 wherein the residue at site 39H has been substituted.

23. The humanized variable domain of claim 1 wherein the residue at site 43H has been substituted.

24. The humanized variable domain of claim 1 wherein the residue at site 45H has been substituted.

25. The humanized variable domain of claim 1 wherein the residue at site 69H has been substituted.

26. The humanized variable domain of claim 1 wherein the residue at site 70H has been substituted.

27. The humanized variable domain of claim 1 wherein the residue at site 74H has been substituted.

28. The humanized variable domain of claim 1 wherein the residue at site 92H has been substituted.

29. An antibody comprising the humanized variable domain of claim 1.

30. An antibody which binds p185HER and comprises a humanized antibody variable domain, wherein the humanized antibody variable domain comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind p185HER incorporated into a human antibody variable domain, and further comprises a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

31. The antibody of claim 30 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

32. The antibody of claim 30 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

33. The antibody of claim 30 wherein the human antibody variable domain is a consensus human variable domain.

34. The antibody of claim 30 wherein the residue at site 4L has been substituted.

35. The antibody of claim 30 wherein the residue at site 38L has been substituted.

36. The antibody of claim 30 wherein the residue at site 43L has been substituted.

37. The antibody of claim 30 wherein the residue at site 44L has been substituted.

38. The antibody of claim 30 wherein the residue at site 46L has been substituted.

39. The antibody of claim 30 wherein the residue at site 58L has been substituted.

40. The antibody of claim 30 wherein the residue at site 62L has been substituted.

41. The antibody of claim 30 wherein the residue at site 65L has been substituted.

42. The antibody of claim 30 wherein the residue at site 66L has been substituted.

43. The antibody of claim 30 wherein the residue at site 67L has been substituted.

44. The antibody of claim 30 wherein the residue at site 68L has been substituted.

45. The antibody of claim 30 wherein the residue at site 69L has been substituted.

46. The antibody of claim 30 wherein the residue at site 73L has been substituted.

47. The antibody of claim 30 wherein the residue at site 85L has been substituted.

48. The antibody of claim 30 wherein the residue at site 98L has been substituted.

49. The antibody of claim 30 wherein the residue at site 2H has been substituted.

50. The antibody of claim 30 wherein the residue at site 4H has been substituted.

51. The antibody of claim 30 wherein the residue at site 36H has been substituted.

52. The antibody of claim 30 wherein the residue at site 39H has been substituted.

53. The antibody of claim 30 wherein the residue at site 43H has been substituted.

54. The antibody of claim 30 wherein the residue at site 45H has been substituted.

55. The antibody of claim 30 wherein the residue at site 69H has been substituted.

56. The antibody of claim 30 wherein the residue at site 70H has been substituted.

57. The antibody of claim 30 wherein the residue at site 74H has been substituted.

58. The antibody of claim 30 wherein the residue at site 75H has been substituted.

59. The antibody of claim 30 wherein the residue at site 76H has been substituted.

60. The antibody of claim 30 wherein the residue at site 78H has been substituted.

61. The antibody of claim 30 wherein the residue at site 92H has been substituted.


63. A humanized antibody which lacks immunogenicity compared to a non-human parent antibody upon repeated administration to a human patient in order to treat a chronic disease in that patient, wherein the humanized antibody comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprises an amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

64. A humanized variant of a non-human parent antibody which binds an antigen and comprises a human variable domain comprising the most frequently occurring amino acid residues at each location in all human immunoglobulins of a human heavy chain immunoglobulin subgroup wherein amino acid residues forming Complementarity Determining Regions (CDRs) thereof comprise non-human antibody amino acid residues, and further comprises a Framework Region (FR) substitution wherein the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) introduces a glycosylation site which affects the antigen binding affinity; (d) participates in the V_\_H-V_\_L interface by affecting the proximity or orientation of the V_\_H and V_\_L regions with respect to one another.

65. The humanized variant of claim 64 which binds the antigen up to 3-fold more in the binding affinity than the parent antibody binds antigen.

66. A humanized antibody heavy chain variable domain comprising non-human Complementarity Determining Regions (CDRs) thereof comprise non-human antibody amino acid residues, and further comprises a Framework Region (FR) substitution wherein the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) introduces a glycosylation site which affects the antigen binding affinity; (d) participates in the V_\_H-V_\_L interface by affecting the proximity or orientation of the V_\_H and V_\_L regions with respect to one another.

67. The humanized variant of claim 65 which binds the antigen up to 3-fold more in the binding affinity than the parent antibody binds antigen.
Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 24H, 73H, 76H, 78H, and 93H, utilizing the numbering system set forth in Kabat.

67. The humanized variable domain of claim 66 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

68. The humanized variable domain of claim 66 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

69. The humanized variable domain of claim 66 wherein the human antibody variable domain is a consensus human variable domain.

70. The humanized variable domain of claim 66 wherein the residue at site 24H has been substituted.

71. The humanized variable domain of claim 66 wherein the residue at site 73H has been substituted.

72. The humanized variable domain of claim 66 wherein the residue at site 76H has been substituted.

73. The humanized variable domain of claim 66 wherein the residue at site 78H has been substituted.

74. The humanized variable domain of claim 66 wherein the residue at site 93H has been substituted.

75. The humanized variable domain of claim 66 which further comprises an amino acid substitution at site 71H.

76. The humanized variable domain of claim 66 which further comprises amino acid substitutions at sites 71H and 73H.

77. The humanized variable domain of claim 66 which further comprises amino acid substitutions at sites 71H, 73H and 78H.

78. An antibody comprising the humanized variable domain of claim 66.

79. A humanized variant of a non-human parent antibody which binds an antigen, wherein the humanized variant comprises Complementarity Determining Region (CDR) amino acid residues of the non-human parent antibody incorporated into a human antibody variable domain, and further comprises Framework Region (FR) substitutions at heavy chain positions 71H, 73H, 78H and 93H, utilizing the numbering system set forth in Kabat.

80. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution where the substituted FR residue:

(a) noncovalently binds antigen directly;
(b) interacts with a CDR; or
(c) participates in the $V_L$-$V_H$ interface by affecting the proximity or orientation of the $V_L$ and $V_H$ regions with respect to one another, and wherein the substituted FR residue is at a site selected from the group consisting of: 4L, 38L, 43L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 24H, 36H, 39H, 43H, 45H, 69H, 70H, 73H, 74H, 76H, 78L, 92L and 93L, utilizing the numbering system set forth in Kabat.

81. The humanized variable domain of claim 80 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

82. The humanized variable domain of claim 80 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 88,
Line 63, please delete “63” and insert therefor -- 79 --.

Signed and Sealed this

Third Day of December, 2002

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