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<p>(54) Title: IN VITRO ANTIBODY AFFINITY MATURATION USING ALANINE SCANNING MUTAGENESIS</p> <p>(57) Abstract</p> <p>A method of mutagenizing antibodies to produce modified antibodies, modified antibodies, DNA encoding the modified antibodies as well as diagnostic kits and pharmaceutical compositions comprising the antibodies or DNA are provided.</p>		

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GA	Gabon				

TITLE OF THE INVENTION

IN VITRO ANTIBODY AFFINITY MATURATION USING ALANINE SCANNING
MUTAGENESIS

CROSS-RELATED TO OTHER APPLICATIONS

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This is a continuation of U.S. Serial No. 08/206,076
filed March 4, 1994, now pending.

BRIEF DESCRIPTION OF INVENTION

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A method of mutagenizing antibodies to produce
modified antibodies, modified antibodies, DNA encoding the
modified antibodies as well as diagnostic kits and pharmaceutical
compositions comprising the antibodies or DNA are provided. The
method of the invention is a systematic means to achieve *in vitro*
antibody maturation and uses alanine scanning mutagenesis. The
invention is particularly exemplified with a set of single chain Fv
(scFv) antibodies obtained by this technique. The resulting
antibodies are directed against the V3 loop of HIV gp120, and show
altered off-rates against the antigen compared to the starting
antibody. Of particular interest are the altered antibodies which
show improved (slower) off-rates to the antigen. Observed
improvements have been as high as eleven-fold over wild-type.

SUMMARY OF THE INVENTION

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A method of mutagenizing antibodies to produce
modified antibodies, modified antibodies, DNA encoding the
modified antibodies as well as diagnostic kits and pharmaceutical
compositions comprising the antibodies or DNA are provided.

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1. Alanine-Scanning Mutagenesis. Each of the
27 amino acids in VH CDR3 of scFv P5Q was converted to alanine
by site-directed mutagenesis. *E. coli* clones were induced to express
scFv with IPTG. Single chain Fv, which is targeted to the
periplasmic space by the fd phage gene3 signal sequence, was

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5 extracted with EDTA. Periplasmic extracts were analyzed by BIAcore™, which measures antibody-antigen affinity by surface plasmon resonance (Fägerstam, 1991), and off-rates determined against an HIV gp120 V3 loop peptide. Results of the alanine scan, relative to P5Q, fall into four classes: i) slower off-rate, ii) faster off-rate, iii) no binding, and iv) minor or no change in off-rate. Standard deviation is $\pm 25\%$.

10 Figure 2. Amino Acid Randomization: Position 107. Arginine at position 107 was mutated to all amino acids by site-directed mutagenesis. Single chain Fv extracts were analyzed by BIAcore. Percent change in off-rates is shown relative to P5Q.

15 Figure 3. Amino Acid Randomization: Position 111. Glutamic acid at position 111 was mutated to all amino acids by site-directed mutagenesis. Single chain Fv extracts were analyzed by BIAcore. Percent change in off-rates is shown relative to P5Q.

20 Figure 4. Amino Acid Randomization: Position 112. Aspartic acid at position 112 was mutated to all amino acids by site-directed mutagenesis. Single chain Fv extracts were analyzed by BIAcore. Percent change in off-rates is shown relative to P5Q.

25 Figure 5. Additive Effect of Combining Optimized Residues. A double mutant, containing the optimized residues, was constructed and analyzed by BIAcore. Percent change in off-rates is shown relative to P5Q.

Figure 6. Nucleotide and amino acid sequences of scFv P5Q with c-myc tail.

DETAILED DESCRIPTION OF THE INVENTION

30 The gp120 V3 domain of human immunodeficiency virus-1 (HIV-1) is a disulfide-linked closed loop of approximately 30 amino acids. The loop, in either native or synthetic form, binds to and elicits anti-HIV-1 antibodies.

The present invention relates to modified antibodies and methods of making modified. The invention is exemplified with modified HIV-1 immunoglobulins and methods of making these

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modified HIV-1 immunoglobulins. The modified immunoglobulins of the present invention contain an altered complementary determining region 3 (CDR3) of HIV-1 neutralizing antibody.

5 The present invention also comprises a method of treating of preventing infection through the administration of a modified antibody to a suitable host. In one embodiment of the invention, the treatment or prevention of HIV infection through the administration of the modified HIV-1 immunoglobulin is described.

10 The present invention also comprises diagnostic kits useful for the detection or characterization of an antigen. Reagents for the kits may include DNA molecules encoding the modified antibodies or the modified antibodies or combinations thereof.

15 A method of mutagenizing antibodies to produce modified antibodies, modified antibodies, DNA encoding the modified antibodies as well as diagnostic kits and pharmaceutical compositions comprising the antibodies or DNA are provided. The method of the invention is a systematic means to achieve *in vitro* antibody maturation and uses alanine scanning mutagenesis. The invention is particularly exemplified with a set of single chain Fv (scFv) antibodies obtained by this technique. The resulting antibodies are directed against the V3 loop of HIV gp120, and show altered off-rates against the antigen compared to the starting antibody. Of particular interest are the altered antibodies which show improved (slower) off-rates to the antigen. Observed improvements have been as high as eleven-fold over wild-type.

25 Maturation was achieved through an alanine scan of complementary determining region 3 (CDR3) to identify positions critical to antigen binding. Critical positions were then randomized to identify amino acids that provided the slowest off-rates. Finally, clones were optimized through the combining of mutations.

30 The underlying principle of the method is the physical and chemical neutrality of alanine. Alanine is substituted throughout a stretch of amino acids, and its effects on binding (such as off-rate and on-rate) are evaluated using conventional methods. The number

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of positions likely to be identified in this manner is relatively small. Once identified, these key positions may be randomized to all amino acids to identify the best amino acid solution at the position. Because all manipulations and evaluations are conducted *in vitro*,
5 physiological bias is limited.

Present methods of *in vitro* antibody maturation are essentially random procedures in which the researcher generates clones with amino acid substitutions and evaluates them. The
10 problem is that the number of substitutions necessary for a thorough evaluation is extremely large. For example, if one were to evaluate all random substitutions in CDR3, a region typically twenty-five residues in length, one would have to examine $9 \cdot 10^{27}$ possibilities. This is beyond the capabilities of present technologies.

Alanine scanning maturation enables the rapid
15 identification of residues most likely to be important in binding. Using the example of a twenty-five residue stretch cited above, only twenty-five substitutions would be necessary. From this initial screen, amino acid positions likely to be critical to binding may be identified. The critical residues may then be randomized to identify
20 the amino acids that optimize binding. Using this method, scFv antibodies with dissociation rates greater than ten-fold slower than the original scFv have been created.

Previous work in *in vitro* antibody maturation used one
25 of two general approaches. In one approach, PCR recombination is used to substitute all or part of the VH and VL genes into libraries of scFv clones. In the second approach, random mutations are made throughout a CDR region of a scFv clone by the use of degenerate oligonucleotides. In both cases, clones were expressed as a phage fd gene 3 fusion surface protein. Higher affinity clones were identified
30 using a panning assay followed by clonal purification of the phage.

Each approach has drawbacks. The PCR method is cumbersome, limited to the sequences of the B cell population, is essentially random in nature, and may introduce unwanted mutations through the PCR recombination step. The randomization approach

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produces only a small fraction of the possible CDR changes. Neither approach allows immediate determination of changes in binding affinity because it is necessary to first generate an enriched population of suitable clones through panning. Both approaches
5 detect only changes which result in improved binding; they do not identify positions for which the change weakened the binding. The latter class of change may include critical binding residues in which the appropriate amino acid solutions leads to improvement.

The method disclosed herein is systematic, thorough and
10 unlikely to introduce unexpected or undesired mutations. All manipulations are done *in vitro*, which minimizes bias due to selection steps. Evaluation of clones is quantitative. In some cases, a key amino acid position may display poorer binding with alanine, but subsequent randomization may yield an amino acid solution which
15 enables improved binding. Such mutations would not be detected by previous methods. Because the method of the present invention does not require phage expression for panning, the method can be used on scFVs, Fabs, and full length antibodies. Use is not restricted to a
20 scFv for phage expression. Using the approach of the present invention, an anti-HIV V3 loop antibody was improved approximately eleven-fold.

Alanine scanning maturation of antibodies is a general method which may be used to improve binding of antibodies to their
25 cognate antigens. The method has been used to identify critical residues in the scFv 447 which can be introduced into MAb447. Such changes may lead to significant improvement of the binding affinity of MAb447 against multiple species of HIV gp120 isolates. This improvement may increase the neutralization capability of the
30 antibody, and significantly lower the effective dose.

Although the method and antibodies of the present invention are exemplified with scFv antibodies, it is readily apparent to those skilled in the art that the method may be used with other types of antibodies or with antibodies targeted against different

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epitopes or antigens. Other types of antibodies include but are not limited to fragments of antibodies and full-length antibodies.

The molecular biology and immunological techniques of the present invention can be performed by standard techniques well-known in the art. See, for example, in Maniatis, T., Fritsch, E.F.,
5 Sambrook, J., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982).

Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore,
10 the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides.

The cloned DNA molecules obtained may be expressed by cloning the gene encoding the altered antibody into an expression vector containing a suitable promoter and other appropriate
15 transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant modified antibodies. Techniques for such manipulations are well-known in the art.

In order to simplify the following Examples and the Detailed Description, certain terms will be defined.

20 Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells and animal cells.
25 Expression vectors include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Specifically designed vectors allow the shuttling of DNA between hosts, such as bacteria-yeast or bacteria-animal cells.

DNA encoding antibodies may also be cloned into an
30 expression vector for expression in a host cell. Host cells may be prokaryotic or eukaryotic, including but not limited to bacteria, yeast, mammalian and insect cells and cell lines.

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to

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transformation, transfection, protoplast fusion, and electroporation.

Expression of cloned DNA may also be performed using *in vitro* produced synthetic mRNA. Synthetic mRNA can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with micro-injection into frog oocytes being preferred.

It is also well-known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences which contain alternative codons which code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variant.

The following examples are provided to further define the invention without, however, limiting the invention to the particulars of these examples.

EXAMPLE 1

Construction of mutations

Plasmid pP5Q was the starting vector for all mutagenic studies. Plasmid pP5Q is a derivative of p5H7 (Cambridge Antibodies). Plasmid pP5Q contains the VH and VL regions originally derived from MAb 447 (Gorney *et al.*) cloned as a single chain fragment variable (scFv).

Table 1 lists some of the oligonucleotide primers used for site-directed mutagenesis of complementary determining region 3 (CDR3) of MAb447. Primers were synthesized on either a model 381A DNA Synthesizer (Applied Biosystems, Foster City, CA) or a Cyclone™ Plus DNA Synthesizer (MilliGen/Biosearch, Marlborough, MA). Mutagenesis was performed with the Transformer™ Mutagenesis Kit (CLONTECH, Palo Alto, CA)

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according to the manufacturer's instructions. All mutations were verified by DNA sequencing using the Sequenase® V2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH).

5

Table 1

Primers:

Randomization of position 107:

10 CTC GGA GAC TCC C/GNN AAT CAT AAT AAA

Randomization of position 111:

GTA GTA GTA GTC C/GNN GGA GAC TCC CCG

Randomization of position 112:

15 GTC GTT GTA GTA GTA GTA GTA C/GNN CTC GGA GAC

EXAMPLE 2

20 Preparation of extracts and BIAcore analysis of scFv Extracts:

Mutagenized plasmids were introduced by electroporation into bacterial strain *Escherichia coli* TG1 for expression. Single colonies were inoculated into 10 ml of 2X-YT (which contains per liter of water 16 g tryptone, 10 g yeast extract and 5 g sodium chloride) supplemented with 2% glucose. Cells were
25 grown overnight at 30°C with vigorous shaking, collected by centrifugation in a Beckman GPR centrifuge at 2500 rpm, and resuspended in 10 ml of fresh 2X-YT supplemented with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to induce expression. Cells were incubated at 30°C for an additional 5–6 hours with
30 vigorous shaking, collected by centrifugation, resuspended in 1 ml of phosphate buffered saline: ethylenediametetraacetic acid (PBS:EDTA; 10 mM sodium phosphate pH7.0, 150 mM sodium chloride 1 mM EDTA), and incubated on ice for 30 minutes to

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release periplasmic proteins. Extracts were clarified by centrifugation and stored at 4°C until use.

EXAMPLE 3

5

Off-rate determinations of the scFv antibodies were determined using the BIAcore system (Pharmacia Biosensor). HIV gp120 V3 loop peptides, Al-1 variant (Ala-1 peptide) were covalently immobilized on a carboxylated dextran/gold matrix via the primary amino group. The carboxyl-dextran matrix was first
10 activated with N-ethyl-N'-(3-diethylaminopropyl)carbodiimide (EDC) and reacted with N-hydroxysuccinimide (NHS). HIV gp120 V3 loop peptides such as Ala-1 peptide were covalently immobilized via the free thiol of a cysteine placed at the N-terminus. These
15 peptides were reacted with the EDC-NHS activated matrix which had been reacted with 2-(2-pyridinyldithio)ethaneamine. Remaining unreacted NHS-ester groups were displaced by addition of ethanolamine. EDTA extracts were added in a flow passing over the immobilized antigen. The refractive index changes, in the form of
20 the surface plasmon resonance caused by the binding and subsequent dissociation of the scFv, were monitored continuously. Off-rates were calculated from the automatically collected data using the Pharmacia Kinetics Evaluation software.

25

EXAMPLE 4

Alanine scanning of CDR3 identifies residues which modulate scFv-antigen binding

30

Alanine scanning mutagenesis was used to identify residues within the VH CDR3 region of scFv clone P5Q critical for binding. It was hypothesized that effects on binding by alanine substitution would lead to four broad classes of effect: class i) slower off-rate; class ii) faster off-rate; class iii) loss of binding; and class iv) minor or no change in off-rate. Class i) and ii) were

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operationally defined as critical. Class iii) was defined as obligatory. Class iv) was defined as noncritical.

5 The 27 positions that comprise VH CDR3 of scFv clone P5Q were individually changed to alanine by site-directed mutagenesis. Periplasmic extracts were prepared from the alanine replacement clones and assayed for off-rate determinations against the AL-1 gp120 V3 loop peptide (Fig. 1). Alanine substitutions at positions 107 and 111 resulted in 1.7 and 2.7 fold improvements in off-rate, respectively. These positions (class i) were judged critical and subsequently randomized to identify optimal residues. Alanine substitutions at positions 102, 112, 113, 114, and 118 led to faster off-rates (class ii); two of these positions were selected for further evaluation. Alanine substitution at positions 98, 101, 115, 116, 117, and 121 resulted in no binding (class iii). Alanine substitution at the remaining fourteen positions had only a minor effect on the off-rate (class iv). The class iii and iv positions were not evaluated further.

EXAMPLE 5

20 Randomization at critical positions to identify optimal amino acid solutions

The two critical class i) positions (107 and 111) were individually randomized to all amino acids, and off-rates against the AL-1 peptide determined. In addition, two class ii) positions (112 and 118) were also selected for randomization studies.

25 The results for position 107 are shown in Fig. 2. The slowest off-rate was observed with the negatively-charged glutamic acid, which decreased dissociation 2.5-fold. Substitution of other polar and charged amino acids had no significant effect on dissociation. With the exception of alanine, substitution with hydrophobic amino acid resulted in complete loss of binding. These results are consistent with the preponderance of surface ligand-contact residues being hydrophilic.

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Randomization of position 111 (Fig. 3) showed that the aromatic residues tyrosine and tryptophan produced the slowest off-rates (dissociation rates decreased 4.2 and 4.7-fold, respectively). However, substitution with any hydrophobic amino acids increased affinity relative to wild-type clone P5Q.

Class ii) positions 112 and 118 (faster off-rate upon alanine substitution) were also selected for amino acid randomization. For both position 112 (Fig. 4) and 118, the residues present in the original scFv P5Q, aspartic acid and asparagine, were the best solutions.

EXAMPLE 6

Improvements at positions 107 and 111 are additive

A double mutant that combined the optimized residues at positions 107 (E) and 111 (W) was constructed to determine whether or not the individual improvements are additive. Figure 5 shows that the double mutant has an off-rate 9-fold slower than wild-type clone P5Q. The off-rate value approximates the product of the fold improvements observed with the individual optimized residues (2.5 for 107E and 4.7 for 111W). One interpretation of this result is that for these two positions, the contributions to scFv-antigen affinity are independent and additive.

EXAMPLE 7

Method of making modified antibodies

An antibody is mutagenized by alanine scanning mutagenesis to produce a modified antibody. The binding of the modified antibody to its antigen is determined. Binding determinations may be made by conventional methods and include off-rate measurements. Modified antibodies having desired characteristics are selected and maintained.

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EXAMPLE 8Method of using modified antibodies

5 The modified antibodies or pharmaceutical compositions thereof are used for the prophylactic or therapeutic treatment of diseases caused by their antigen. Methods of treatment include, but are not limited to, intravenous or intraperitoneal injection of the modified antibody.

10

EXAMPLE 9Diagnostic kit employing modified antibodies

15 The modified antibodies of Example 7 are used as reagents in diagnostic kits. The modified antibody reagents may be further modified through techniques which are well-known in the art, such as radiolabeling or enzyme-labeling. The diagnostic kit may be used to detect or characterize the antigens.

20

EXAMPLE 10DNA encoding modified antibodies

25 The DNA encoding the modified antibody of Example 7 is used as a reagent for the production of modified antibodies. The DNA may be incorporated into an expression vector. The expression vector may be used to transform a host cell. Cultivation of the host cell under conditions suitable for the expression results in the production of modified antibody.

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EXAMPLE 11DNA encoding modified antibodies

5 The DNA encoding the modified antibody of Example 7 is used to detect DNA encoding the antigen in test samples. Methods of detection include, but are not limited to, hybridization under selective conditions. Test samples include, but are not limited to, samples of blood, cells, and tissues.

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EXAMPLE 12Preparation of modified light chain immunoglobulins

15 The light chain of an immunoglobulin is mutagenized by alanine scanning mutagenesis to produce a modified immunoglobulin having modified binding characteristics. The modified immunoglobulin is used as a reagent for diagnostic kits or as a therapeutic agent.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: LEWIS, CRAIG M.
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HOLLIS, GREGORY F.
- (ii) TITLE OF INVENTION: IN VITRO ANTIBODY MATURATION
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 - (D) STATE: NJ
 - (E) COUNTRY: USA
 - (F) ZIP: 07065
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
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 - (A) APPLICATION NUMBER: US 08/206,079
 - (B) FILING DATE: 04-MAR-1994
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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 - (B) REGISTRATION NUMBER: 36,090
 - (C) REFERENCE/DOCKET NUMBER: 19190P
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 - (B) TELEFAX: (908) 594-4720

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 816 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

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

GCCATGGCCG AGGTGCAGCT GGTGGAGTCT GGGGGAGGCT TGGTAAAGCC TGGGGGGTCC 60
 CTCAGACTCA CCTGTGTAGC CTCTGGCTTC ACGTTCAGTG ATGTCTGGCT GAACTGGGTC 120
 CGCCAGGCC CAGGGAAGGG GCTGGAGTGG GTCGGCCGTA TTAAAAGCGC CACTGATGGT 180
 GGGACAACAG ACTACGCTGC ATCCGTGCAA GGCAGATTCA CCATCTCAAG AGATGACTCA 240
 AAAAACACGC TATATCTGCA AATGAATAGC CTGAAAACCG AGGACACAGC CGTTTATGCC 300
 TGCAACACAG ATGGTTTTAT TATGATTCCG GGAGTCTCCG AGGACTACTA CTACTACTAC 360
 AACGACGTTT GGGGCAAAGG GACCACGGTC ACCGTCTCCT CAGGTGCAGG CGGTTCAGGC 420
 GGAGGTGGCT CTGGCGGTGG CGGATCGCAG TCTGTGTTGA CGCAGCCGCC CTCAGTGTCT 480
 GCGGCCCCAG GACAGAAGGT CACCATCTCC TGCTCTGGAA GCAGCTCCAA CATTGGGAAT 540
 AATTATGTAT TGTGGTACCA GCAGTTCCCA GGAACAGCCC CCAAATCCTT CATTATGGC 600
 AATAATAAGC GACCCTCAGG GATTCCTGAC CGATTCTCTG GCTCCAAGTC TGGCACGTCA 660
 GCCACCCTGG GCATCACCGG ACTCCAGACT GGGGACGAGG CCGATTATTT CTGCGCAACA 720
 TGGGATAGCG GCCTGAGTGC TGATTGGGTG TTCGGCGGAG GGACCAAGCT GACCGTCCTA 780
 GGTGCGGCCG CAGAACAAA ACTCATCTCA GAAGAG 816

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 272 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Ala Met Ala Glu Val Glx Leu Val Glu Ser Gly Gly Gly Leu Val Lys
 1 5 10 15
 Pro Gly Gly Ser Leu Arg Leu Thr Cys Val Ala Ser Gly Phe Thr Phe
 20 25 30
 Ser Asp Val Trp Leu Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 35 40 45
 Glu Trp Val Gly Arg Ile Lys Ser Ala Thr Asp Gly Gly Thr Thr Asp
 50 55 60

- 16 -

Tyr Ala Ala Ser Val Gln Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser
 65 70 75 80
 Lys Asn Thr Leu Tyr Leu Glx Met Asn Ser Leu Lys Thr Glu Asp Thr
 85 90 95
 Ala Val Tyr Ser Cys Asn Thr Asp Gly Phe Ile Met Ile Arg Gly Val
 100 105 110
 Ser Glu Asp Tyr Tyr Tyr Tyr Tyr Tyr Asn Asp Val Trp Gly Lys Gly Thr
 115 120 125
 Thr Val Thr Ala Ser Ser Gly Ala Gly Gly Ser Gly Gly Gly Gly Ser
 130 135 140
 Gly Gly Gly Ser Gln Ser Val Leu Thr Gln Pro Pro Ser Val Ser Ala
 145 150 155 160
 Ala Pro Gly Gln Lys Val Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn
 165 170 175
 Ile Gly Asn Asn Tyr Val Leu Trp Tyr Gln Gln Phe Pro Gly Thr Ala
 180 185 190
 Pro Lys Leu Leu Ile Tyr Gly Asn Asn Lys Arg Pro Ser Gly Ile Pro
 195 200 205
 Asp Arg Phe Ser Gly Ser Lys Leu Leu Ile Tyr Gly Ala Thr Leu Gly
 210 215 220
 Ile Thr Gly Leu Gln Thr Gly Asp Gln Ala Asp Tyr Phe Cys Ala Thr
 225 230 235 240
 Trp Asp Ser Gly Leu Ser Ala Asp Trp Val Phe Gly Gly Gly Thr Lys
 245 250 255
 Leu Thr Val Leu Gly Ala Ala Ala Glu Gln Lys Leu Ile Ser Glu Glu
 260 265 270

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WHAT IS CLAIMED IS:

- 5 1. A DNA molecule encoding a modified antibody, the modified antibody being derived from a native antibody by alanine scanning mutagenesis and the modified antibody having binding characteristics different than binding characteristics of the native antibody.
- 10 2. The DNA molecule of Claim 1 wherein the native antibody is MAb447.
- 15 3. The DNA molecule of Claim 2, the DNA molecule being selected from the group consisting of P5Q, DNA encoding modified antibodies of Figures 1, 2, 3, 4, 5, combinations thereof, derivatives thereof and degenerate variants thereof.
- 20 4. A method of modifying an antibody to make a modified antibody comprising replacing at least one amino acid of the antibody with alanine to produce a modified antibody.
- 25 5. The method of Claim 4 wherein the modified antibody has improved binding characteristics.
- 30 6. Modified antibodies produced by the method of Claim 4 or homologues thereof.
7. The method of Claim 4 wherein the antibody is MAb447.
8. The method of Claim 7 wherein the amino acid replaced with alanine is located in complementary determining region 1, complementary determining region 2 or complementary determining region 3 of MAb447.

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9. The modified antibodies of Claim 6 selected from the group consisting of P5Q, the antibodies of Figures 1, 2, 3, 4, 5, combinations thereof, derivatives thereof, and homologues thereof.

5

10. Diagnostic kits comprising the modified antibodies produced by the method of Claim 6.

10

11. Diagnostic kits comprising the DNA molecules of Claim 1.

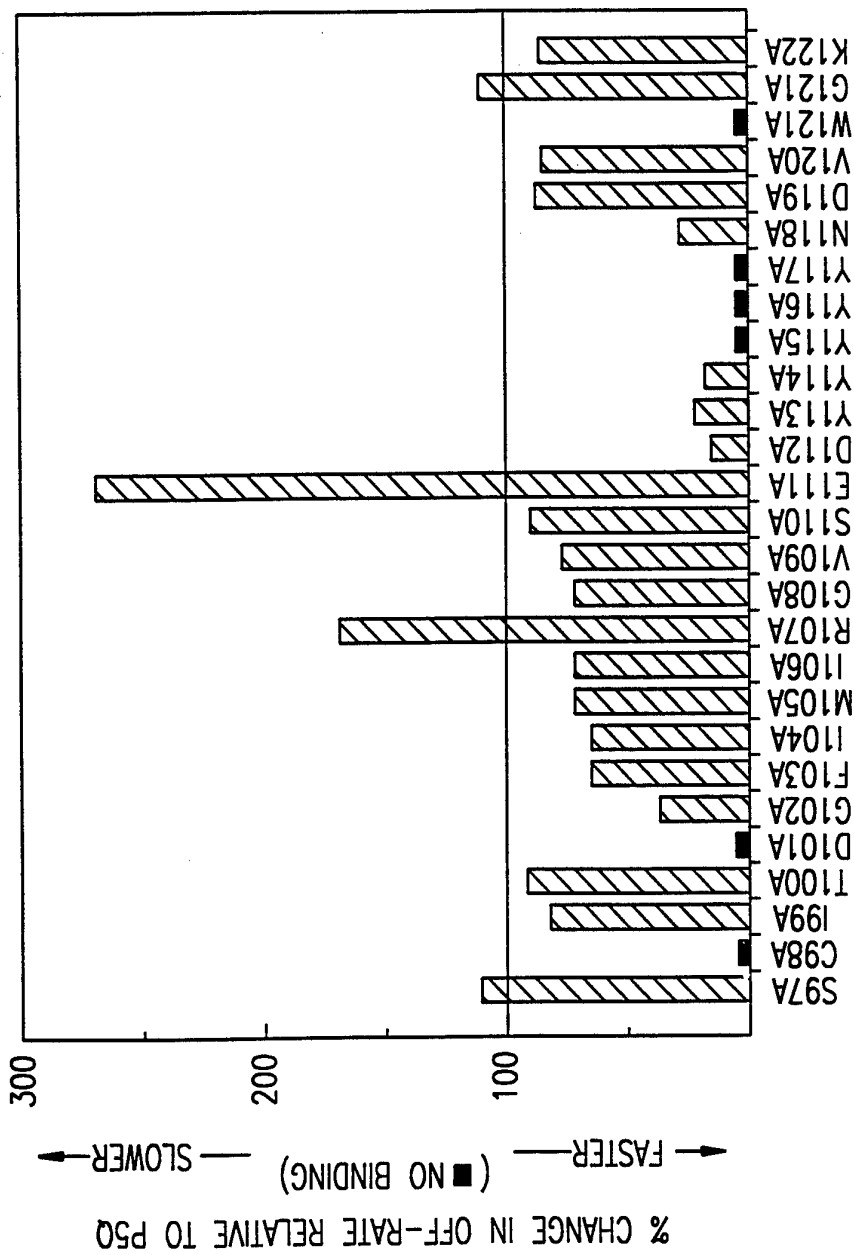
15

12. A pharmaceutical composition comprising at least one modified antibody of Claim 6 or DNA encoding at least one modified antibody of Claim 6 or combinations thereof.

20

25

30



AMINO ACID POSITIONS

FIG. 1

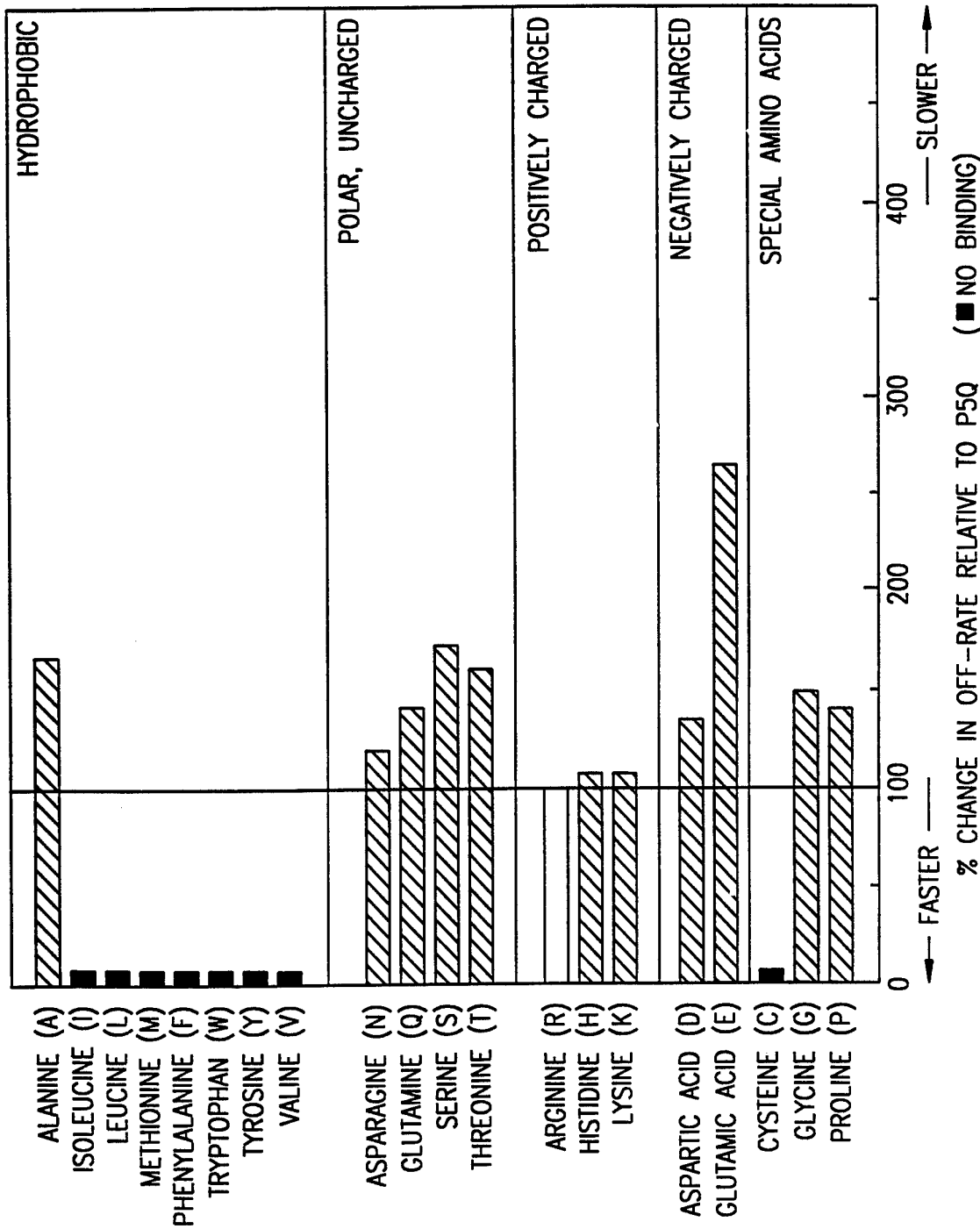


FIG.2

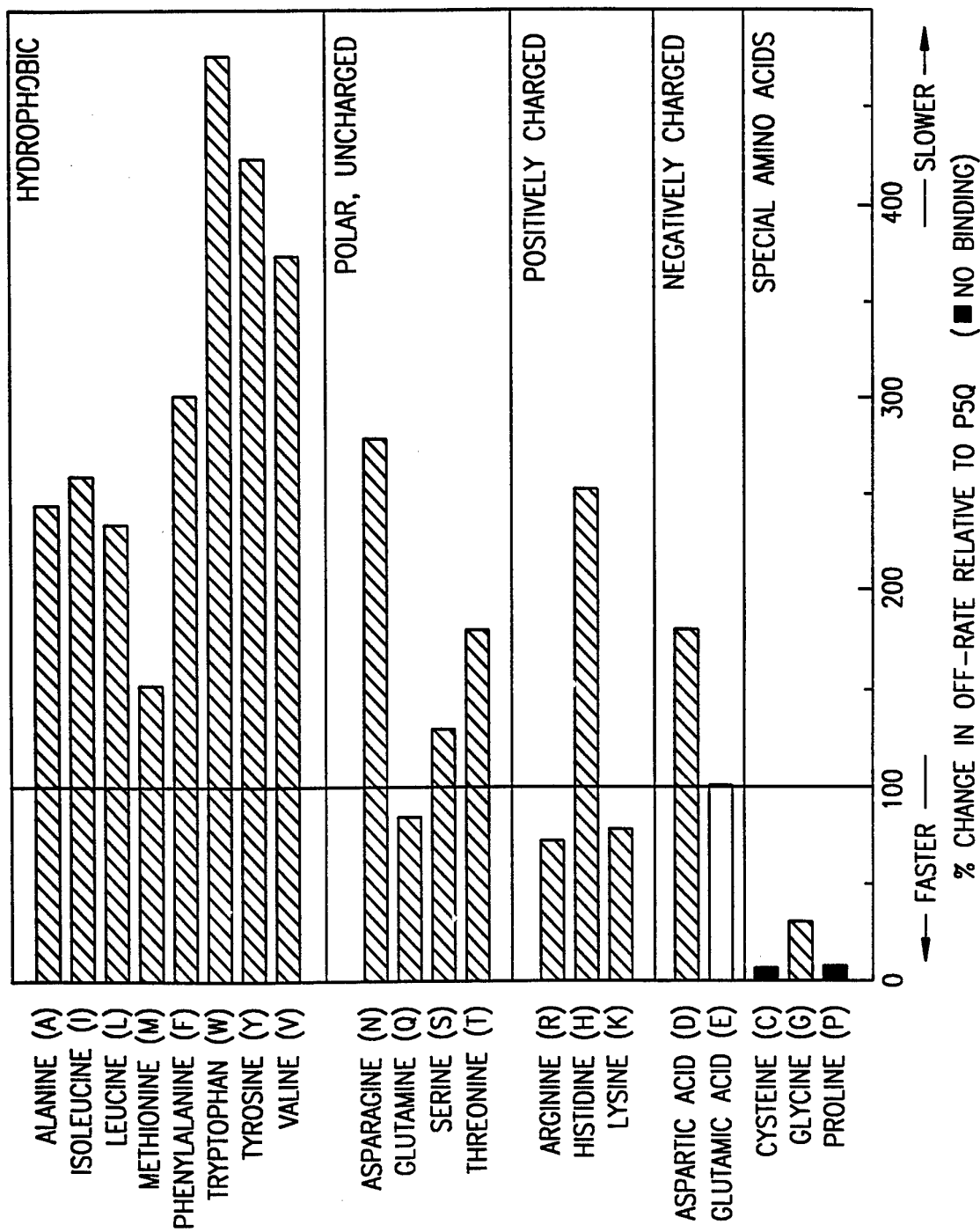


FIG.3

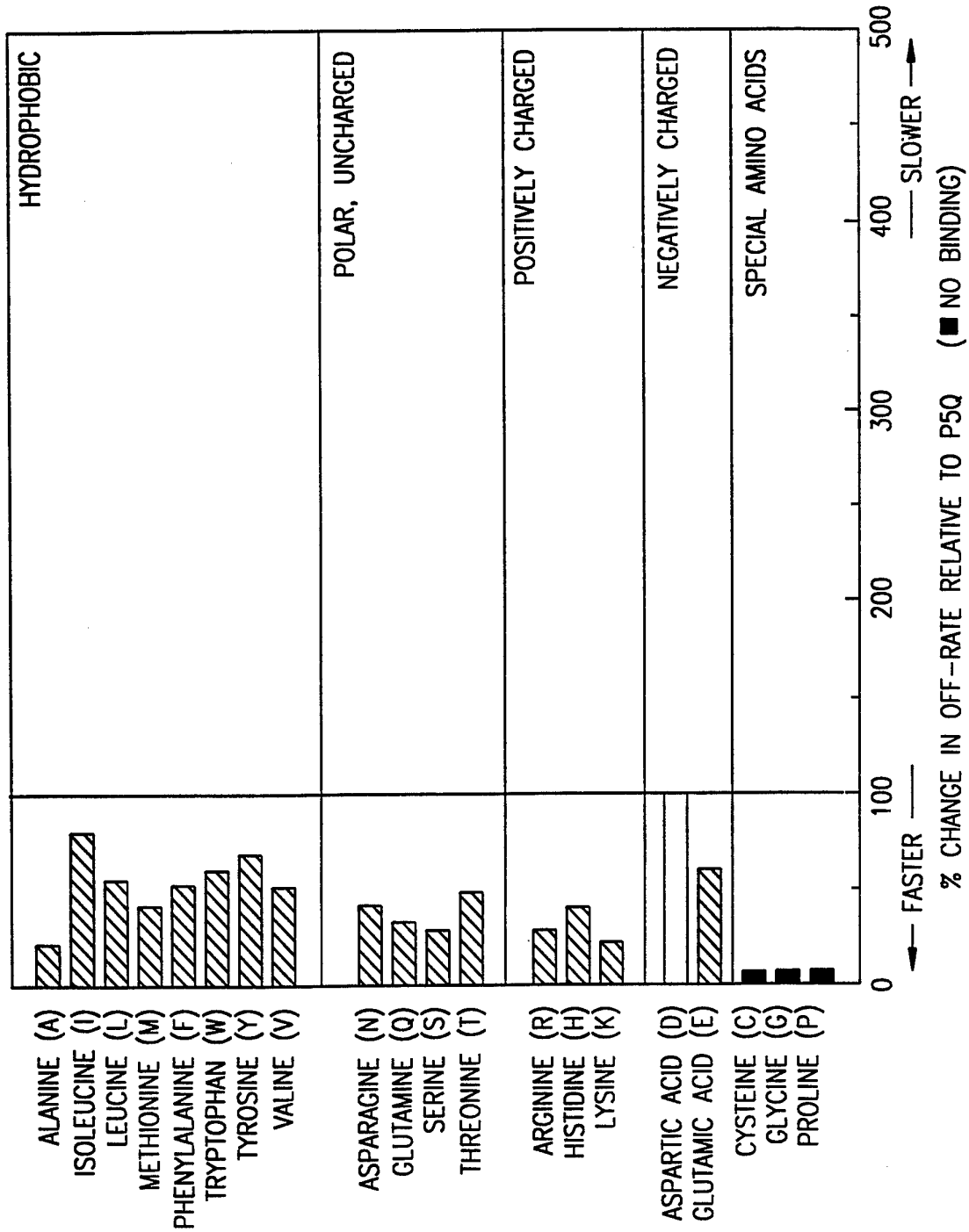


FIG.4

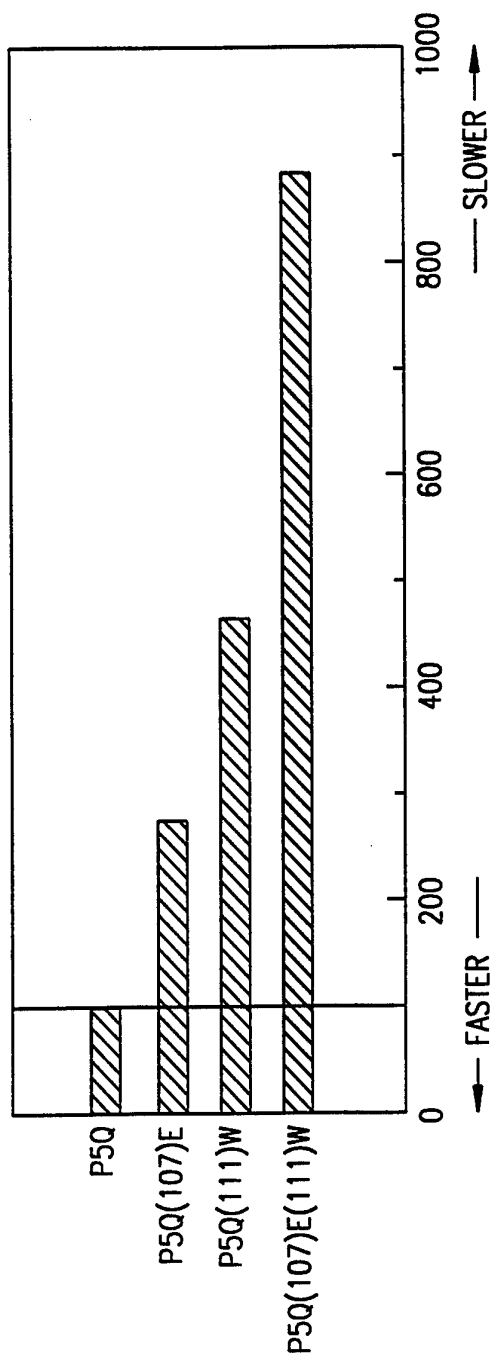


FIG.5

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10	*	20	*	30	*	40	*	50	*	60	*								
GCC	ATG	GCC	GAG	GTG	CAG	CTG	GTG	GAG	TCT	GGG	GGA	GGC	TTG	GTA	AAG	CCT	GGG	GGG	TCC
Ala	Met	Ala	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Lys	Pro	Gly	Gly	Ser
70	*	80	*	90	*	100	*	110	*	120	*								
CTC	AGA	CTC	ACC	TGT	GTA	GCC	TCT	GCC	TTC	ACG	TTC	AGT	GAT	GTC	TGG	CTG	AAC	TGG	GTC
Leu	Arg	Leu	Thr	Cys	Val	Ala	Ser	Gly	Phe	Thr	Phe	Ser	Asp	Val	Trp	Leu	Asn	Trp	Val
130	*	140	*	150	*	160	*	170	*	180	*								
CGC	CAG	GCC	CCA	GGG	AAG	GGG	CTG	GAG	TGG	GTC	GGC	CGT	ATT	AAA	AGC	GCC	ACT	GAT	GGT
Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val	Gly	Arg	Ile	Lys	Ser	Ala	Thr	Asp	Gly
190	*	200	*	210	*	220	*	230	*	240	*								
GGG	ACA	ACA	GAC	TAC	GCT	GCA	TCC	GTG	CAA	GGC	AGA	TTC	ACC	ATC	TCA	AGA	GAT	GAC	TCA
Gly	Thr	Thr	Asp	Tyr	Ala	Ala	Ser	Val	Gln	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asp	Ser

FIG.6a

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250	*	260	*	270	*	280	*	290	*	300	*
AAA AAC ACG CTA TAT CTG CAA ATG AAT AGC CTG AAA ACC GAG GAC ACA GCC GGT TAT TCC											
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Lys Thr Glu Asp Thr Ala Val Tyr Ser											
310	*	320	*	330	*	340	*	350	*	360	*
TGC AAC ACA GAT GGT TTT ATT ATG ATG CCG GGA GTC TCC GAG GAC TAC TAC TAC TAC TAC											
Cys Asn Thr Asp Gly Phe Ile Met Ile Arg Gly Val Ser Glu Asp Tyr Tyr Tyr Tyr Tyr											
370	*	380	*	390	*	400	*	410	*	420	*
AAC GAC GTT TGG GGC AAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GCA GCC GGT TCA GGC											
Asn Asp Val Trp Gly Lys Gly Thr Thr Val Thr Ala Ser Ser Gly Ala Gly Gly Ser Gly											
430	*	440	*	450	*	460	*	470	*	480	*
GGA GGT GGC TCT GGC GGT GGC GGA TCG CAG TCT GTG TTG ACG CAG CCG CCC TCA GTG TCT											
Gly Gly Gly Ser Gly Gly Gly Ser Gln Ser Val Leu Thr Gln Pro Ser Val Ser											

FIG.6b

490	*	500	*	510	*	520	*	530	*	540	*									
GCG	GCC	CCA	GGA	CAG	AAG	GTC	ACC	ATC	TCC	TGC	TCT	GGA	AGC	AGC	TCC	AAC	ATT	GGG	AAT	
Ala	Ala	Pro	Gly	Gln	Lys	Val	Thr	Ile	Ser	Cys	Ser	Gly	Ser	Ser	Asn	Ile	Gly	Asn		
550	*	560	*	570	*	580	*	590	*	600	*									
AAT	TAT	GTA	TTG	TGG	TAC	CAG	CAG	TTC	CCA	GGA	ACA	GCC	CCC	AAA	CTC	CTC	ATT	TAT	GGC	
Asn	Tyr	Val	Leu	Leu	Trp	Tyr	Gln	Gln	Phe	Pro	Gly	Thr	Ala	Pro	Lys	Leu	Ile	Tyr	Gly	
610	*	620	*	630	*	640	*	650	*	660	*									
AAT	AAT	AAG	CGA	CCC	TCA	GGG	ATT	CCT	GAC	CGA	TTC	TCT	GCC	TCC	AAG	TCT	GGC	ACG	TCA	
Asn	Asn	Lys	Arg	Pro	Ser	Gly	Ile	Pro	Asp	Arg	Phe	Ser	Gly	Ser	Lys	Ser	Gly	Thr	Ser	
670	*	680	*	690	*	700	*	710	*	720	*									
GCC	ACC	CTG	GGC	ATC	ACC	GGA	CTC	CAG	ACT	GGG	GAC	GAG	GCC	GAT	TAT	TTC	TGC	GCA	ACA	
Ala	Thr	Leu	Gly	Ile	Thr	Gly	Ile	Leu	Gln	Thr	Gly	Asp	Glu	Ala	Asp	Tyr	Phe	Cys	Ala	Thr

FIG.6c

730	*	740	*	750	*	760	*	770	*	780	*										
TGG	GAT	AGC	GGC	CTG	AGT	GCT	GAT	TGG	GTG	TTC	GGC	GGA	GGG	ACC	AAG	CTG	ACC	GTC	CTA		
TIP	Asp	Ser	Gly	Leu	Ser	Ala	Asp	Trp	Val	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Thr	Val	Leu		
790	*	800	*	810	*																
GGT	GCG	GCC	GCA	<u>GAA</u>	<u>CAA</u>	<u>AAA</u>	<u>CTC</u>	<u>ATC</u>	<u>TCA</u>	<u>GAA</u>	<u>GAG</u>										
Gly	Ala	Ala	Ala	Glu	Gln	Lys	Leu	Ile	Ser	Glu	Glu										

FIG.6d

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02492

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 16/00, 16/46; A61K 39/00; C12N 15/12, 15/13
US CL :424/133.1, 144.1; 536/23.53; 530/387.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/133.1, 144.1; 536/23.53; 530/387.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

SEQUENCE SEARCH, MEDLINE, EMBASE, LIFESCI, BIOSYS, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. IMMUNOLOGY, VOL. 150, NO. 2, ISSUED 15 JANUARY 1993, M.K. GORNY ET AL., "REPertoire OF NEUTRALIZING HUMAN MONOCLONAL ANTIBODIES SPECIFIC FOR THE V3 DOMAIN OF HIV-1 GP120", PAGES 635-643, SEE ENTIRE DOCUMENT.	1-12
Y	PROC. NATL. ACAD. SCI. USA, VOL. 87, ISSUED SEPTEMBER 1990, A. ASHKENAZI ET AL., "MAPPING OF THE CD4 BINDING SITE FOR HUMAN IMMUNODEFICIENCY VIRUS BY ALANINE-SCANNING MUTAGENESIS", PAGES 7150-7154, SEE ENTIRE DOCUMENT.	1-12
Y	SCIENCE, VOL. 244, ISSUED 02 JUNE 1989, B.C. CUNNINGHAM ET AL., "HIGH-RESOLUTION EPITOPE MAPPING OF hGH-RECEPTOR INTERACTIONS BY ALANINE-SCANNING MUTAGENESIS", PAGES 1081-1085, SEE ENTIRE DOCUMENT.	1-12

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 MAY 1995

Date of mailing of the international search report

23 MAY 1995

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