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- (71) Applicant (for all designated States except US): **THE SCRIPPS RESEARCH INSTITUTE** [US/US]; 10550 North Torrey Pines Road, La Jolla, CA 92037 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **BURTON, Dennis, R.** [US/US]; 6044 Beaumont Avenue, La Jolla, CA 92037 (US). **WILSON, Ian** [GB/US]; 1025 Newkirk Drive, La Jolla, CA 92037 (US). **PANTOPHLET, Ralph** [NL/US]; 7564 Charmant Drive, Apt. 1518, San Diego, CA 92122 (US).
- (74) Agents: **FITTING, Thomas et al.**; The Scripps Research Institute, 10550 North Torrey Pines Road, TPC-8, La Jolla, CA 92037 (US).
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(54) Title: IMMUNOGENIC MUTANT HUMAN IMMUNODEFICIENCY VIRUS GP120 POLYPEPTIDES, AND METHODS OF USING SAME

(57) Abstract: Immunogenic mutant HIV gp120 polypeptides that contain at least one mutation in at least one epitope that specifically binds non-neutralizing antibodies are provided. Also provided are methods of making such HIV neutralizing antibodies using such immunogenic mutant gp120 polypeptides. In addition, methods of inducing an HIV neutralizing antibody response in a subject are provided.



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**IMMUNOGENIC MUTANT HUMAN IMMUNODEFICIENCY VIRUS gp120
POLYPEPTIDES, AND METHODS OF USING SAME**

[0001] This invention was made in part with government support under Grants No. AI 33292 awarded by the National Institutes of Health. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of immunology and specifically to human monoclonal antibodies which bind and neutralize human immunodeficiency virus (HIV), e.g., HIV type I (HIV-1), and immunogens useful for inducing neutralizing antibodies against an HIV such as HIV-1.

BACKGROUND INFORMATION

[0003] HIV is the focus of intense studies as it is the causative agent for acquired immunodeficiency syndrome (AIDS). Immunotherapeutic methods are one of several approaches to prevention, cure or remediation of HIV infection and HIV-induced diseases. Specifically, the use of neutralizing antibodies in passive immunotherapies is of central importance to the present invention. Passive immunization of HIV-1 infected humans using human sera containing polyclonal antibodies immunoreactive with HIV has been reported (see, for example, Jackson et al., Lancet, Sep. 17:647-652, (1988); Karpas et al., Proc. Natl. Acad. Sci., USA, 87:7613-7616 (1990)).

[0004] Numerous groups have reported the preparation of human monoclonal antibodies that neutralize HIV isolates *in vitro*. The described antibodies typically have immunospecificities for epitopes on the HIV glycoprotein gp160 or the related glycoproteins gp120 or gp41 (see, for example Karwowska et al., AIDS Research and Human Retroviruses, 8:1099-1106 (1992); Takeda et al., J. Clin. Invest., 89:1952-1957 (1992); Tilley et al., AIDS Research and Human Retroviruses, 8:461-467 (1992); Laman et al., J. Virol., 66:1823-1831 (1992); Thali et al., J. Virol., 65:6188-6193 (1991); Ho et al., Proc. Natl. Acad. Sci., USA, 88:8949-8952 (1991); D'Souza et al., AIDS, 5:1061-1070 (1991); Tilley et al., Res. Virol., 142:247-259 (1991); Broliden et al., Immunol., 73:371-376 (1991); Posner et al., J. Immunol., 146:4325-4332 (1991); and Gorny et al., Proc. Natl.

Acad. Sci., USA, 88:3238-3242 (1991)). Levy provides a review of pathogenesis of HIV infection and therapeutic modalities including the use of passive immunity with anti-HIV antibodies (Microbiol. Rev., 57:183-289 (1993)).

[0005] The majority of human monoclonal antibodies reported to date are not effective in passive immunization therapies. Further, as monoclonal antibodies, they all each react with an individual epitope on the HIV envelope surface glycoproteins, gp120 or gp160, or against the V3 loop of gp120 or against the envelope transmembrane glycoprotein, gp41. The epitope against which an effective neutralizing antibody immunoreacts has not been identified.

[0006] There continues to be a need to develop human monoclonal antibody preparations with significant HIV neutralization activity. In addition, there is a need for monoclonal antibodies immunoreactive with additional and diverse neutralizing epitopes on HIV gp120. Additional (new) epitope specificities are required because, upon passive immunization, the administered patient can produce an immune response against the administered antibody, thereby inactivating the particular therapeutic antibody. Furthermore, the well documented ability of HIV to mutate its envelope glycoprotein structure and thereby alter its reactivity with the immune system of an infected host produces variant "field isolates" which compromise the utility of individual antibody preparations immunoreactive with an individual laboratory strain of HIV. Existing antibody preparations tend to be less potent against primary field isolates of HIV than against laboratory strains (Moore et al., Perspectives in Drug Discovery and Design, 1:235-250 (1993)).

[0007] Broadly neutralizing antibodies can protect against intravenous and mucosal challenge with immunodeficiency viruses in animal models (4, 38, 50, 65, 68, 88, 96, 98, 125). It has, therefore, become increasingly clear that eliciting such antibodies should be a major goal in efforts to develop a human immunodeficiency virus type 1 (HIV-1) vaccine (18, 21, 66, 85, 117, 145). The animal model studies provide a number of guidelines as to the types of antibody that should be elicited. First, protection is generally provided by antibodies that effectively neutralize virus *in vitro* (88, 95). Second, serum neutralizing

antibody levels at the time of virus challenge need to be relatively high (around 1:100) to achieve sterile protection, although lower levels can provide benefit in terms of delayed and/or decreased viremia (88, 98, 125). Third, protection by broadly neutralizing human monoclonal antibodies (mAbs) against a number of viruses suggests that protection against many different strains of HIV-1 may be achievable (4, 97, 98).

[0008] The major problem to date, from a vaccine standpoint, is that no immunogen has been generated that can elicit reasonable levels of such broadly neutralizing antibodies. These antibodies should be targeted to relatively conserved and exposed regions of the HIV-1 envelope, but the paucity of broadly neutralizing antibodies in natural infection suggests that the virus presents these regions to the immune system in such a way as to minimize an effective antibody response (21, 100, 143, 145). A molecular understanding of regions on the HIV-1 envelope that are exposed and conserved and how they can be recognized by antibodies would be invaluable in the design of immunogens that can elicit broadly neutralizing antibodies.

[0009] The CD4 binding site (CD4bs) on the HIV-1 surface glycoprotein gp120 is a highly conserved region that is known to be exposed for ligand binding (30, 54). In theory, this would seem to form an excellent target for neutralizing antibodies. Many mAbs that bind with high affinity to the CD4bs of monomeric gp120 from various primary and T-cell line-adapted (TCLA) HIV-1 isolates have been isolated (see, hypertext transfer protocol ([http](http://resdb.lanl.gov/ABDB/antibody_id.htm)), at URL "resdb.lanl.gov/ABDB/antibody_id.htm", which is incorporated herein by reference). These mAbs are characterized by their ability to compete with soluble CD4 and with one another (80).

[0010] Anti-CD4bs mAbs typically neutralize TCLA viruses with moderate efficacy, but neutralize primary isolates of HIV-1 very weakly if at all (102). However, one mAb, b12, which interacts with the CD4bs, does neutralize many primary and TCLA viruses very efficiently (22, 29, 53, 69). MAb b12 and non-neutralizing anti-CD4bs mAbs typically have very similar binding affinities for monomeric gp120 from a number of isolates (78, 80). The differences between b12 and the other mAbs in neutralizing activity against TCLA

viruses, therefore, have been associated with different affinities for the mature envelope trimer expressed on virions (35, 110, 116). Typically, mAb b12 is able to bind with comparable affinity to monomeric gp120 and mature trimer on the surface of infected cells (99), which is believed to be identical to the functional envelope molecule on the surface of virions (116). Non-neutralizing anti-CD4bs mAbs, on the other hand, bind with lowered affinity to the mature trimer. The implication, therefore, is that b12 is able to bind similarly to monomer gp120 and to the native TCLA trimer and neutralize the virus effectively, whereas the other anti-CD4bs mAbs suffer some impediment in their access to the CD4bs on the mature TCLA trimer and, therefore, neutralize virus less effectively (103). Lower levels of envelope expression have made the investigation of the correlation between binding to mature trimer and neutralization more troublesome for primary HIV-1. Such a correlation has been reported (121) and, as disclosed herein, it was considered that the explanation for the efficacy of b12 against primary viruses can be similar to that for TCLA viruses.

SUMMARY OF THE INVENTION

[0011] The present invention is based on the seminal discovery that although monomeric gp120 binds b12 and non-neutralizing anti-CD4bs antibodies equivalently, monomeric gp120 can be mutated such that it can bind neutralizing antibodies such as b12 to a greater extent that it can bind non-neutralizing mAbs. As disclosed herein, such mutated gp120 polypeptides are useful as immunogens for stimulating an antibody response primarily comprising HIV neutralizing antibodies, including neutralizing antibodies specific for HIV type I (HIV-1), HIV type 2 (HIV-2), or both, particularly HIV-1. Accordingly, immunogenic mutant gp120 polypeptides that can stimulate a neutralizing antibody response are provided, as are methods of using such immunogenic mutant gp120 polypeptides, and antibodies raised using such immunogenic gp120 polypeptides.

[0012] The invention provides immunogenic modified (mutant) HIV-1 gp120 polypeptides, which can elicit an antibody response in a subject, particularly an antibody response characterized by the generation of neutralizing HIV-1 antibodies, including a much greater level of neutralizing antibody (as compared to non-neutralizing antibody) than

that elicited by a wild-type gp120 polypeptide. Preferably, the neutralizing antibody response is elicited in the absence of a non-neutralizing antibody response. In one aspect, wild-type gp120 polypeptide was modified by the substitution of at least one alanine for the wild-type amino acid residue(s). In another aspect, variable loop deletions were made in the gp120 molecule.

[0013] The present invention relates to an immunogenic mutant gp120 polypeptide that can stimulate a neutralizing antibody response against a human immunodeficiency virus (HIV), wherein the mutant gp120 polypeptide comprises an HIV (e.g., HIV-1) gp120 polypeptide having at least one amino acid mutation in at least one epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody. As such, an immunogenic mutant gp120 polypeptide of the invention can contain 2, 3, 4, 5, 6, 7, or more two amino acid mutations in 1, 2, 3, 4, 5, 6, or more epitopes of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody. Thus, an immunogenic mutant gp120 polypeptide can have, for example, at least a first amino acid mutation in a first epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody, and at least a second amino acid mutation in a second epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody, wherein the first epitope and the second epitope can be the same or different. The epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody can include any epitope, including, for example, an epitope comprising Phe43 cavity perimeter amino acid residues, V1 loop amino acid residues, V2 loop amino acid residues, V3 loop amino acid residues, non-neutralizing face amino acid residues, C1 domain amino acid residues, C5 domain amino acid residues, co-receptor binding site amino acid residues, or a combination thereof.

[0014] The amino acid mutation (or mutations) introduced into in epitopes bound by a non-neutralizing antibody generally is a substitution of a first amino acid residue for a second different amino acid residue, which can be a conservative substitution or a non-conservative substitution. In one embodiment, amino acid mutations in epitopes bound by a non-neutralizing antibody are exemplified by the substitution of an amino acid residue of HIV-1 gp120 polypeptide with an alanine residue, for example, a substitution such as

G473A, D474A, M475A, R476A, or a combination thereof, e.g., D474A and R476A; G473A and M475A; D474A, M475A and R476A; or G473A, D474A, M475A and R476A; or a substitution such as D113A, V127A, D180A, N197A, or S256A, or any combination of the above-described mutations.

[0015] In another embodiment, the amino acid mutations in epitopes bound by a non-neutralizing antibody are exemplified by substitutions, including one or two substitutions, that generate a glycosylation motif (e.g., an *N*-glycosylation motif) at a position in the HIV-1 gp120 polypeptide that otherwise is not subject to glycosylation. Such substitutions are exemplified by H92N and N94T; Q114N and L115T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; or I423N and N425T, and combinations thereof. For example, an immunogenic mutant HIV-1 gp120 polypeptide can have Q114N, L115T, S143T, E150N, and G152T substitutions and, if desired, can have one or more substitutions, e.g., H92N; K171N and Y173T; and/or I423N.

[0016] In still another embodiment, the amino acid mutations in epitopes bound by a non-neutralizing antibody are exemplified by substitutions that generate a glycosylation motif and, further, substitutions that do not generate a glycosylation motif, particularly substitutions that decrease the ability of a non-neutralizing antibody to bind an HIV-1 gp120 epitope without substantially affecting the ability of a neutralizing antibody to bind an HIV-1 gp120 epitope. An immunogenic mutant gp120 polypeptide containing such combinations of mutations is exemplified by a gp120 polypeptide containing one or more of G473A, D474A, M475A, and R476A substitutions and one or more of H92N, Q114N, L115T, S143T, E150N, G152T, K171N, Y173T, and I423N substitutions, including an immunogenic mutant gp120 polypeptide containing all of these substitutions. As disclosed herein, the mutations of an epitope bound by a non-neutralizing antibody also can be a deletion, for example, a deletion of C1 and/or C5 amino acid residues.

[0017] The present invention also provides neutralizing antibodies stimulated by a mutant HIV gp120 immunogen. Such antibodies, which can be in an antiserum or an immunoglobulin fraction of such a serum or substantially purified antibodies, are useful for

diagnostic and/or immunotherapeutic purposes. In one aspect, the antibodies have a specificity that is substantially similar to the b12 monoclonal antibody (U.S. Pat. Nos. 5,652,138 and 5,804,440, each of which is incorporated herein by reference), particularly HIV neutralizing activity. As disclosed herein, the b12 monoclonal antibody provides an exemplary neutralizing antibody that is useful for identifying an immunogenic mutant HIV-1 gp120 polypeptide of the invention.

[0018] The present invention further provides a method of generating neutralizing antibodies for HIV in a subject. Such a method can utilize an immunogenic mutant HIV-1 gp120 polypeptide of the invention as an immunogen in a subject, thereby generating an antibody response comprising primarily or preferentially HIV-1 neutralizing antibodies. The subject can be any subject capable of raising an immune response against such an immunogen, including a mammalian subject, particularly a human subject. For example, the invention provides a method of inducing antibodies that can neutralize HIV by immunizing a subject with an immunogenic mutant HIV gp120 polypeptide that can stimulate a neutralizing antibody response against an HIV as disclosed herein, e.g., an HIV-1 gp120 polypeptide having at least one amino acid mutation in at least one epitope of the gp120 polypeptide specifically bound by a non-neutralizing antibody. The epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody can include, for example, Phe43 cavity perimeter amino acid residues, V1 loop amino acid residues, V2 loop amino acid residues, V3 loop amino acid residues, non-neutralizing face amino acid residues, C1 domain amino acid residues, C5 domain amino acid residues, co-receptor binding site amino acid residues, or a combination of such epitopes.

[0019] The mutation (or mutations) in the epitope (or epitopes) can be a substitution or deletion, as disclosed herein, including, for example, one, two or three amino acid mutations that generate a glycosylation motif in the epitope. The immunogenic mutant gp120 polypeptide used in a method of the invention can be any immunogenic mutant gp120 polypeptide that preferentially induces an HIV neutralizing antibody response, for example, an immunogenic mutant HIV-1 gp120 polypeptide containing D113A; V127A; D180A; N197A; S256A; G473A; D474A; M475A; R476A; H92N and N94T; Q114N and L115T;

S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or a combination thereof (e.g., an immunogenic mutant gp120 polypeptide containing G473A, D474A, M475A, R476A, and, optionally, H92N and N94T; Q114N and L115T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; or I423N and N425T; or a combination thereof).

[0020] A method of inducing an HIV neutralizing antibody response in a subject can further include obtaining antiserum from the subject, wherein the antiserum comprises HIV neutralizing antibodies. In one embodiment, the antiserum contains HIV-1 neutralizing antibodies. In another embodiment, the subject is a human subject. Such a method can further include an HIV neutralizing antibody fraction from said antiserum and/or substantially purifying HIV neutralizing antibodies from the subject or from antiserum obtained from the subject. Accordingly, antiserum containing HIV neutralizing antibodies, an isolated HIV neutralizing antibody fraction, and a substantially purified HIV neutralizing antibody, which can be a polyclonal or monoclonal antibody (e.g., an anti-HIV-1 neutralizing monoclonal antibody) obtained by a method of the invention is provided.

[0021] The present invention also provides methods of producing HIV neutralizing antibodies by contacting antibody producing cells with an immunogenic mutant HIV gp120 polypeptide of the invention. In one embodiment, the method is performed, for example, by contacting antibody producing cells (e.g., spleen cells) in culture under conditions such that antibody producing cells in the culture are induced to express HIV neutralizing antibodies (e.g., HIV-1 neutralizing antibodies). Such a method can further include isolating the HIV neutralizing antibodies from the culture. Accordingly, the invention provides isolated HIV neutralizing antibodies produced by such a method, for example, isolated HIV-1 neutralizing antibodies.

[0022] In another embodiment, a method of producing HIV neutralizing antibodies is performed, for example, by administering the immunogenic mutant gp120 polypeptide to a subject under conditions sufficient for the immunogenic mutant gp120 polypeptide to stimulate an immune response in the subject. Such a method can further include obtaining

blood, plasma, and/or serum from the subject, and/or obtaining antiserum containing the HIV neutralizing antibodies. In addition, the method can further include isolating an HIV neutralizing antibody fraction and/or substantially purifying HIV neutralizing antibodies from the antiserum, or from blood, plasma or serum of the subject. The method can also include isolating antibody producing cells from the subject, wherein the cells can be used as a source of the HIV neutralizing antibodies, for example, by immortalizing the antibody producing cells or generating hybridomas from the antibody producing cells, thus further providing a means to obtain monoclonal HIV neutralizing antibodies. Accordingly, the invention provides antiserum, an isolated HIV neutralizing antibody fraction; substantially purified HIV neutralizing antibodies produced by such methods, isolated antibody producing cells produced by such methods, and antibodies produced by such isolated antibody producing cells or cells derived therefrom.

[0023] The present invention also provides a method of treating a human subject having or at risk of having an HIV infection or HIV-induced disease. Such a method can be performed, for example, by administering to the subject a therapeutically effective amount of an immunogenic mutant gp120 polypeptide of the invention, thereby actively inducing a neutralizing antibody response in the subject, and treating the infection or disease. Such a method can be useful as a prophylactic method, thus reducing the likelihood that a subject can become infected with HIV, or as a therapeutic for a subject infected with HIV, thus providing a means to ameliorate HIV infection.

[0024] The present invention also relates to a method of ameliorating an HIV infection in a subject. In one embodiment, the method is performed, for example, by administering an immunogenic mutant gp120 polypeptide of the invention to the subject. The immunogenic mutant gp120 polypeptide can be any immunogenic mutant gp120 polypeptide that preferentially induces an HIV neutralizing antibody response, including, for example, an immunogenic mutant HIV-1 gp120 polypeptide comprises D474A and R476A; G473A and M475A; D474A, M475A and R476A; or G473A, D474A, M475A and R476A, which, optionally, can further comprise H92N and N94T; Q114N and L115T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; or I423N and

N425T; or a combination thereof, and/or a deletion of C1 domain amino acid residues, C5 domain amino acid residues, or a combination thereof, which can be useful for ameliorating an HIV-1 infection in a subject.

[0025] In another embodiment, a method of ameliorating an HIV infection in a subject is performed, for example, by administering HIV neutralizing antibodies to the subject, wherein the HIV neutralizing antibodies comprise antibodies stimulated in response to mutant gp120 polypeptide of the invention. The HIV neutralizing antibodies can be obtained using any method as disclosed herein, including, for example, by immunizing an individual with the immunogenic mutant gp120 polypeptide, and isolating the HIV neutralizing antibodies, or antiserum containing the antibodies, for the individual; or by immunizing an individual with the immunogenic mutant gp120 polypeptide, thereafter generating hybridomas from antibody producing cells from the individual, and isolating the HIV neutralizing antibodies from hybridomas expressing the HIV neutralizing antibodies.

[0026] The present invention further relates to a method of reducing or preventing HIV infection in a subject. Such a method, which can be a prophylactic method, can be performed, for example, by administering an immunogenic mutant gp120 polypeptide of the invention to the subject under conditions that stimulate an HIV neutralizing antibody response in the subject.

[0027] The present invention further provides a method for identifying a neutralizing antibody effective for neutralizing HIV or related viruses. The method includes a competition assay whereby an immunogenic mutant gp120 polypeptide is contacted with a b12 or b12-like antibody, followed by contacting with a test neutralizing antibody, particularly a monoclonal antibody. Such an assay also can be performed in reverse, wherein the test antibody is first contacted with the gp120 molecule, followed by contacting with the b12 antibody. A desired neutralizing antibody of the invention can be identified by detecting competition of a test antibody with a b12 antibody for gp120 binding, thereby indicating that the test antibody is a neutralizing antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] Figures 1A to 1D show the location of amino acid substitutions on gp120, the spatial relationship of epitopes on gp120, and variability of gp120 residues among primate immunodeficiency viruses and HIV-1. HIV gp120 is exemplified by the gp120 of HIV-1 isolates JR-CSF (GenBank Acc. No. AAB03749; for encoding nucleic acid, see GenBank Acc. No. M38429, each of which is incorporated herein by reference) and JR-FL (AAB05604; for encoding nucleic acid, see GenBank Acc. No. U63632, each of which is incorporated herein by reference). See also Appendix 5, Figures 1-15 in black and white.

[0029] Figure 1A provides a ribbon amino acid sequence diagram of gp120_{JR-CSF} indicating the locations of the alanine substitutions in this study. Arrows indicate amino acids that were mutated to alanine. Variable loop deletions are indicated by white letters encircled by blue spheres. Light blue dots indicate the positions of amino acid mutations in b12 neutralization escape mutants (74, 104). Red and yellow dots indicate primary and secondary b12 contact residues, respectively, based on a computational docking model of b12 and core gp120_{HxB2} (90). Primary b12 contacts are gp120 residues that, based on the docking model, contact mAb b12; secondary contacts are gp120 residues within 3-5 Å of mAb b12.

[0030] Figure 1B shows the location of mutations (colored blue) mapped onto the gp120 core of HxB2. The view is from the perspective of CD4.

[0031] Figure 1C shows the approximate location of the faces of the gp120 core, defined by the interaction of gp120 and antibodies (61, 143, 145). The region in the CD4bs that is accessible to neutralizing ligands on primary HIV-1 isolates (i.e. mAbs CD4-IgG2 and b12), termed neutralizing face, is shown in yellow. The region of gp120 that is believed to be poorly accessible on oligomeric gp120 and elicits non-neutralizing antibodies is shown in cyan. The location of the immunologically 'silent' face, which encompasses the epitope recognized by the broadly neutralizing mAb 2G12 (115, 119, 137), is shown in magenta. The co-receptor binding site is shown in light grey. Modeled carbohydrate chains are shown in dark grey and black. The approximate areas that are believed to be covered by the

V2 and V3 loops (primarily the coreceptor binding site) are indicated. The locations of the Phe43 cavity (61), involved in CD4 binding, and the V1/V2 loop are also indicated.

[0032] Figure 1D shows the molecular surface of gp120 depicting the sequence variability of the amino acid residues among primate immunodeficiency viruses and HIV-1: green, residues conserved among all primate immunodeficiency viruses; yellow, residues conserved among all HIV-1 isolates but not among all primate immunodeficiency viruses; grey, residues that are variable among HIV-1 isolates. Amino acid conservation defined as in (61).

[0033] Figures 2A to 2C show the apparent affinity of mAbs for alanine mutants of gp120_{JR-CSF} relative to wild-type gp120. Numbering is based on the sequence of HIV-1_{HxB2} (HxB2) (57). The amino acid sequences for gp120 of HIV-1 isolates JR-CSF (GenBank Acc. No. AAB03749; for encoding nucleic acid, see GenBank Acc. No. M38429, each of which is incorporated herein by reference; see Appendices 1 and 2, respectively) and JR-FL (GenBank Acc. No. AAB05604; for encoding nucleic acid, see GenBank Acc. No. U63632, each of which is incorporated herein by reference; see Appendices 3 and 4, respectively) are provided for comparison, wherein, for example, P313-X-R315 corresponds to positions 309-311 in GenBank Acc. No. AAB03749 and to positions 308-310 in GenBank Acc. No. AAB05604. On the x-axis, only every second amino acid residue listed in Table 1 (see below) is numbered. Red bars represent CD4 binding. A schematic of the conserved and variable regions of HIV-1 gp120 is also shown below. Numbers indicate amino acid residues (HxB2 numbering). C, conserved domain; V, variable region.

[0034] Figure 2A shows antibody b3 binding (green bars).

[0035] Figure 2B shows antibody b6 binding (grey bars).

[0036] Figure 2C shows antibody b12 binding (blue bars).

[0037] Figure 3 shows the effects of alanine substitutions on antibody binding mapped onto the HxB2 gp120 core structure. The view is from the perspective of CD4. Only substitutions that affect antibody binding are colored and labeled. Alanine substitution of

residues that are colored yellow significantly enhanced mAb binding (>200% affinity relative to wild type), whereas those colored blue significantly reduced mAb or CD4 binding (<50% affinity relative to wild type). Amino acid substitutions in the V2 loop that affected antibody or CD4 binding are indicated by colored circles on the left of each structure.

[0038] Figure 4 provides epitope maps of the unique effects of alanine substitutions on mAb binding affinity. Color scheme and labeling as in the legend to Figure 3, except that, in this figure, amino acid mutations which did not significantly affect antibody binding are also indicated (colored black). Top panel: differential map of alanine point mutations for which the effect on mAb binding was unique as compared to the other two mAbs. Bottom panel: differential map of the unique effects of alanine substitutions on binding by mAbs b3 and b6, respectively, in comparison to b12.

[0039] Figure 5 depicts alanine mutants that were selected for neutralization assays. Labeled amino acids that are colored grey indicate mutant pseudovirions for which there was a reasonable correlation between b12 binding to monomeric gp120 and neutralization efficiency. Residues labeled and colored yellow indicate pseudovirions, which were neutralized equally well or better than wild-type virus despite a decrease in b12 affinity for monomeric gp120 of the respective mutant. Residues labeled and colored red indicate pseudovirions which were neutralized less well than wild-type virus despite an increase in b12 binding affinity for monomeric gp120 for the respective mutant.

[0040] Figures 6A to 6D show antibody binding in the context of the functional envelope trimer.

[0041] Figure 6A depicts the trimeric gp120 model as proposed by Kwong et al. (62); gp120 is depicted as viewed from the virus.

[0042] Figure 6B shows a docking model of mAb b12 (yellow) to gp120.

[0043] Figures 6C and 6D depict how two hypothetical non-neutralizing anti-CD4bs mAbs (pink, Figure 6C; and green, Figure 6d) can interact with gp120. Note how b12 and

the non-neutralizing anti-CD4bs mAbs are able to interact with monomeric gp120, but that only b12 binds at an orientation that also allows the interaction with gp120 in the context of a functional envelope trimer. For clarity, only the antibody Fab fragments are shown.

[0044] Figures 7A to 7D show binding of CD4 binding site (CD4bs) mAbs (left) and non-CD4bs mAbs (right) to gp120_{JR-FL} of mutant GDMR in ELISA. Supernatants containing monomeric gp120 were captured onto ELISA plate wells and probed with varying concentrations of mAb, starting at 10 µg/ml. Bound antibody was detected with alkaline phosphatase-conjugated secondary antibody. Absorbance was measured at 405 nm.

[0045] Figures 7A and 7B show antibody binding to wild-type gp120.

[0046] Figures 7C and 7D show antibody binding to gp120 of mutant GDMR.

[0047] Figure 8A to 8D show binding of V3 loop mAbs to wild-type gp120_{JR-FL} and glycoprotein of mutant P313N by western blot analysis and in an ELISA.

[0048] Figures 8A and 8B show western blot analyses of wild-type gp120 (lane 1) and mutant glycoprotein (lane 2) reacted with mAb b12 (1 µg/ml; Figure 8A) or with mAbs 19b, 447-52D and loop 2 (pooled mixture; 1 µg/ml each; Figure 8B). Molecular weight indicators (bars) and the average molecular weight of wild type and mutant glycoproteins (arrows) are shown on the left; kDa, kilodaltons.

[0049] Figures 8C and 8D show MAb binding to wild-type gp120 (Figure 8C) and mutant P313N (Figure 8D) as determined by ELISA.

[0050] Figure 9 shows binding of mAb b12 to gp120 with added *N*-glycosylation sequons. Wild-type gp120 and mutant glycoproteins (labeled I-VII) were captured onto ELISA wells and probed with mAb b12 (10 ng/ml). HIVIG (1 µg/ml) was used to ensure that equivalent amounts of protein were captured. Bound antibody was detected with peroxidase-conjugated secondary antibody and absorbance measured at 450 nm.

[0051] Figures 10A to 10C show the location of introduced *N*-glycan attachment sites and alanine substitutions, mapped onto the gp120 core. *N*-linked oligosaccharides (yellow) were modeled onto the core structure (61) according to the most likely glycoforms, as inferred from a previous study (151). The putative locations of the V1 and V2 loops are also indicated in each panel. The 20 Å-bar represents the average width of a typical antibody-combining site. Figures were made with the RasMolTM program (118) and modified using Adobe PHOTOSHOP.

[0052] Figure 10A depicts the gp120 core shown from the perspective of CD4. The attachment sites of the extra glycans are labeled and colored dark blue; dark blue-colored spheres indicate those in the V1 and V2 loops. The alanine substitutions at positions 473-476 (GDMR) are labeled and colored red. The perspectives shown in Figure 10B and 10C are also indicated by arrows.

[0053] Figure 10B shows a view of the gp120 outer domain (61, 143). The spatial location of the V3 and V4 loops, which are proposed to extend from the protein surface, are also indicated.

[0054] Figure 10C shows a view of the proposed gp41-gp120 interface (61, 143).

[0055] Figure 11 shows a determination of the molecular weight of mutant mCHO*-GDMR by western blot. Wild-type gp120_{JR-FL} and mutant glycoprotein were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and probed with a polyclonal antibody preparation generated against the C5 region of gp120 (5 µg/ml). Lane 1, wild type; lane 2, mutant mCHO*-GDMR. Molecular weight indicators are shown on the left and the average molecular weight of each glycoprotein is denoted on the right; kDa, kilodaltons.

[0056] Figures 12A to 12H show binding of anti-gp120 mAbs to wild type (Figures 12A-D, left) and mutant mCHO*-GDMR (Figures 12E-H, right) glycoproteins. Captured glycoproteins were probed with mAb concentrations indicated on the x-axis. Absorbance was measured at 405 nm.

[0057] Figures 12A and 12E show binding of mAbs to the C1, C5, C1-C4, and C1/C5 domains.

[0058] Figures 12B and 12F show binding mAbs to the CD4bs.

[0059] Figures 12C and 12G show binding of mAbs to the coreceptor binding site.

[0060] Figures 12D and 12H show binding of mAbs to the V2 and V3 loops.

[0061] Figure 13 shows glycoproteins of mutants mCHO*-GDMR and mCHO*-GDMR ,N/C (N and C termini deleted), captured onto ELISA plate wells with indicated antibodies (10 µg/ml) and detected with biotinylated mAb 2G12 (1 µg/ml). Grey bars represent mutant mCHO*-GDMR; dark bars represent mutant mCHO*-GDMR ,N/C. Absorbance (optical density, OD) was measured at 450 nm.

[0062] Figure 14 shows the reactivity of HIVIG (open symbols) and mAb b12 (filled symbols) in ELISA with wild-type gp120 and mutant glycoproteins. wt, wild-type gp120; GDMR, mutant GDMR in which residues at positions 473-476 in gp120 have been substituted by alanine (see Example 2); GDMR-P313N, similar to mutant GDMR with the addition of an *N*-glycosylation site in the V3 loop; mCHO*-GDMR, similar to mutant GDMR-P313N, with the addition of six glycosylation motifs (2 in the V1 loop, 1 in the V2 loop, and 3 on the gp120 core).

[0063] Figure 15 shows binding of CD4bs mAbs and mAb A32 to glycoprotein of mutant mCHO*. This mutant contains all the glycosylation sequons that are also present in mutant mCHO*-GDMR, but lacks the alanine substitutions on the edge of the Phe43 cavity.

DETAILED DESCRIPTION OF THE INVENTION

[0064] Irrespective of the underlying mechanisms responsible for neutralization differences, b12 and non-neutralizing anti-CD4bs mAbs constitute probes to distinguish presentations of gp120 that are desirable for vaccine purposes from those that are less desirable. As a first step, alanine scanning mutagenesis was used to identify amino acid residues on HIV-1 gp120 that modulate binding by b12 and to compare these to residues

that affect binding of two representative non-neutralizing anti-CD4bs mAbs that previously were characterized in detail, namely b3 and b6. Amino acid changes that had an effect on antibody binding were compared to those that affected CD4 binding. The results show that the mAbs and CD4 bind gp120 with a similar footprint. The footprint for CD4 was very close to that which would be expected from the crystal structure of a complex of CD4 and the core of gp120 (61). The footprint for antibody b12 also corresponded well with that expected from a docking model of the b12 structure and the gp120 core structure (90). However, a number of differences observed in the epitope maps between b12 and the non-neutralizing anti-CD4bs mAbs suggest that HIV-1 gp120 can be engineered to generate a molecule that is more disposed to eliciting b12-like antibodies.

[0065] Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 C.F.R. 1.822(b)(2)), standard one letter and three letter abbreviations for amino acid residues are used, as shown in the "Table of Correspondence" (below).

[0066] It should be noted that all amino acid residue sequences represented herein by formulae have a left-to-right orientation in the conventional direction of amino terminus to carboxy terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence and modified and unusual amino acids, such as those listed in 37 C.F.R. 1.822(b)(4), and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or a covalent bond to an amino-terminal group such as NH₂ or acetyl or to a carboxy-terminal group such as COOH.

Table of Correspondence:

SYMBOL		
1-Letter/3-Letter/AMINO ACID		

Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
X	Xaa	Unknown or other

[0067] Recombinant DNA (rDNA) molecule: A DNA molecule produced by operatively linking two DNA segments. Thus, a recombinant DNA molecule is a hybrid DNA molecule comprising at least two nucleotide sequences not normally found together in nature. rDNA's not having a common biological origin, i.e., evolutionarily different, are said to be "heterologous".

[0068] Vector: A rDNA molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of genes encoding for one or more polypeptides are referred to herein as "expression vectors". Particularly important vectors allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

[0069] Antibody: The term antibody in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v).

[0070] Antibody Combining Site: An antibody combining site is that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) an antigen. The term "immunoreact" in its various forms means specific binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof.

[0071] Monoclonal Antibody: A monoclonal antibody in its various grammatical forms refers to a population of antibody molecules that contain only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody thus typically displays a single binding affinity for any epitope with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having

a plurality of antibody combining sites, each immunospecific for a different epitope, e.g., a bispecific monoclonal antibody. Although historically a monoclonal antibody was produced by immortalization of a clonally pure immunoglobulin secreting cell line, a monoclonally pure population of antibody molecules can also be prepared by the methods of the present invention.

[0072] Global efforts to obtain an effective vaccine against HIV-1 have thus far failed. The induction of antibodies with broad anti-viral activity, considered a highly beneficial feature of a future vaccine (18, 19, 66, 85, 117, 143, 145), has proven particularly problematic. The use of soluble monomeric HIV-1 gp120, the major component of the viral envelope spike, has yielded antibodies that bind solely to monomeric gp120 or only to a narrow range of HIV-1 isolates (9, 27, 63). The crystal structures of the gp120 core in complex with CD4 and an antibody Fab fragment (60, 61, 143) have shed light on why it may be difficult to elicit antibodies that are capable of recognizing gp120 as presented on the virion surface. Conserved sequences, such as found in the CD4 binding domain, lie recessed within the core and are partially occluded by (hyper)variable loops, which then reduces antibody recognition (61, 143, 145). Furthermore, although other conserved regions, such as the interface between gp120 and the transmembrane unit glycoprotein gp41 (61), can be readily exposed on monomeric gp120, these epitopes are most likely occluded on the envelope spike (143, 145).

[0073] Because of the disappointing results with monomeric gp120, new approaches are being explored for eliciting broadly neutralizing antibodies. Two main approaches are currently being investigated using HIV envelope glycoproteins. One strategy focuses on the preservation or reconstruction of the trimeric envelope spike. For example, virions have been chemically inactivated by modification of the zinc finger domains of the nucleocapsid region, while maintaining the native envelope structure (3, 113). In another approach, soluble gp140 oligomers containing the ectodomain of gp41 covalently linked to gp120 have been generated by fusing GCN4 trimerization domains or T4 bacteriophage fibrin trimeric motifs to the C-terminus of soluble, uncleaved gp140 glycoproteins (147-149). In other studies cysteine residues have been incorporated into gp120 and gp41 (11, 114) to

prevent dissociation of the two subunits through the formation of an intersubunit disulfide bridge upon expression of cleaved gp140. More recently, proteoliposomes have been generated containing native, trimeric uncleaved gp160, CT (cytoplasmic tail-deleted) glycoproteins (44). Although all of these approaches appear promising, such attempts to mimic native HIV envelope trimers have the limitation that key cross-neutralizing epitopes may be of relatively low immunogenicity on the trimer (143, 145).

[0074] A second strategy for obtaining broadly neutralizing antibodies with recombinant envelope glycoproteins focuses on the use of monomeric, but slightly modified, gp160 or gp140 glycoproteins. For example, various envelope glycoproteins have been generated in which the V2 loop has been deleted, with the aim of increasing the exposure of neutralizing epitopes (128). In other studies, partially deglycosylated recombinant gp160 (13), or recombinant viruses expressing gp120 glycosylation mutants, have been generated (107). Unfortunately, all of these approaches have so far failed to provide immunogens that elicit the desired level of neutralizing antibodies (23, 107), most likely because the elicited antibodies are unable to recognize their cognate epitopes on wild-type virus particles.

[0075] Neutralizing antibodies should target conserved regions on the HIV-1 envelope because such antibodies are most likely to be cross-reactive and useful in protection against HIV. The CD4-binding site (CD4bs) on gp120 of HIV-1 is a particularly attractive target for vaccine design because it displays a high degree of conservation (61), and is accessible to neutralizing monoclonal antibodies (mAbs) on the surface of primary HIV-1 isolates prior to CD4 binding (110). One mAb, b12, can be particularly useful as a model for the design of a vaccine capable of inducing potentially neutralizing antibodies targeted to the CD4bs. MA b12 was isolated from a combinatorial phage display library developed from bone marrow donated by an individual who had been HIV positive for more than 6 years, but had not yet developed clinical symptoms (20). MA b12 effectively neutralizes a broad range of primary isolates from various HIV-1 clades as well as T-cell-line-adapted viruses (22, 29, 53). It has been inferred that this capability stems from b12 being able to bind functional oligomeric gp120 with comparable affinity to monomeric gp120 (99, 116, 121); non-neutralizing mAbs bind with substantially lower affinity, or not at all, to functional

oligomeric gp120, presumably due to epitope occlusion or steric hindrance by vicinal gp120 protomers on the viral surface (103, 143, 145).

[0076] Monomeric gp120 thus contains all of the antigenic determinants to elicit a broadly neutralizing antibody such as b12. However, the use of monomeric gp120 is jeopardized by the exposure of non-neutralizing epitopes that are normally occluded on oligomeric gp120 or that reside in the variable regions, in particular the V2 and V3 loops (100, 103, 145). These antigenic determinants are immunologically dominant over more conserved neutralizing epitopes (70, 86). Furthermore, they may induce antibodies that interact with gp120 in a manner that is not permitted on native envelope spikes. As disclosed herein, monomeric HIV-1 gp120 was engineered to restrict antibody binding to broadly neutralizing antibodies, e.g., b12. As a first step, alanine scanning mutagenesis was used to identify residues on gp120 that are important for binding of b12, CD4 and the non-neutralizing CD4bs mAbs b3 and b6 (Pantophlet et al., *J. Virol.* 77:642-658 (2003), which is incorporated herein by reference; see Example 1). Although these three antibodies and CD4 bind monomeric gp120 with a similar footprint, four residues were identified on the perimeter of the Phe43 cavity (61) that, when substituted by alanine, abrogated binding of the two non-neutralizing antibodies and CD4 (94). The binding of another non-neutralizing mAb, F105, was also abolished, whereas that of two further non-neutralizing CD4bs mAbs, F91 and 15e, was reduced. In contrast, the binding of mAb b12 relative to wild type monomeric gp120 was slightly enhanced (94).

[0077] The modified gp120 polypeptides containing alanine substitutions provide promising prospective immunogens. However, considering that the variable loops on monomeric gp120 also can serve as immune decoys (34, 70, 86, 122) gp120 was further altered so as to focus the antibody response on the CD4bs; the concept of refocusing B-cell responses has been postulated by Delves et al. (32), who suggested that epitope-specific molecules could be elicited by selectively mutating 'undesired' epitopes, while preserving the overall fold of the protein and, hence, the desired B-cell (or T-cell) epitope(s). In a recent report (24), the ϵ -chain of human chorionic gonadotrophin was used as a model system to show the feasibility of this strategy. This study showed that a single amino acid

substitution that does not disrupt the overall conformation of the protein is sufficient to shift the immune response away from an unwanted epitope and towards a weakly immunogenic determinant (24).

[0078] Rather than inserting single mutations, a previously described approach (37) was adopted, wherein undesired epitopes are masked through the incorporation of extra *N*-linked glycans. As disclosed herein, this strategy resulted in blocking of the binding of non-neutralizing and weakly neutralizing antibodies, whereas binding of the broadly neutralizing antibody b12 was retained (Example 2). These reengineered HIV-1 gp120 polypeptides provide potential immunogens useful, for example, as a component of an HIV-1 vaccine, and can serve as a basis for alternative approaches to the development of candidate vaccines against other viral pathogens.

[0079] The present invention relates to mutant gp120 molecules, referred to generally herein as immunogenic mutant gp120 polypeptides, which stimulate neutralizing antibodies, including human antibodies that are specific for and neutralize an human immunodeficiency virus (HIV), particularly HIV-1. In an embodiment of the invention, epitopic polypeptide sequences in glycoprotein gp120 of HIV-1 are provided that are capable of stimulating neutralizing antibodies for HIV. In another embodiment, the gp120 immunogen binds only neutralizing antibodies, and not non-neutralizing antibodies. Stimulation of such human monoclonal antibodies with a claimed specificity, and like human monoclonal antibodies with like specificity, are useful in the diagnosis and therapy of HIV-induced disease.

[0080] The term "HIV-induced disease" means any disease caused, directly or indirectly, by HIV. An example of a HIV-induced disease is acquired immunodeficiency syndrome (AIDS) caused by HIV-1 infection, and any of the numerous conditions associated generally with AIDS and caused by HIV infection. Thus, in one aspect, the present invention is directed to modified or mutant gp120 molecules that provide an HIV neutralization site and to monoclonal antibodies stimulated by the invention immunogenic mutant HIV-1 gp120 molecules. The monoclonal antibody referred to as "b12", as disclosed in U.S. Patent Nos. 5,652,138 and 5,804,440, herein incorporated by reference in their entirety, is an

exemplary neutralizing antibody to utilize to compare other antibodies against for purposes of the present invention. Thus, if a modified or mutant gp120 molecule elicits a human monoclonal antibody response and the antibody generated binds and neutralizes HIV, particularly HIV-1, in a manner similar to a b12 human monoclonal antibody then the mutant gp120 molecule is a candidate immunogen for eliciting a neutralizing antibody response.

[0081] A “modified” or “mutant” gp120 molecule is a molecule that has at least one deletion, substitution or the like such that the molecule is different at the amino acid residue level from a wild-type gp120 molecule from which it was derived. It should be understood that gp120 molecules may also naturally differ within a population of molecules. A molecule prior to mutagenesis will be considered “wild-type” for purposes of this invention.

[0082] It is also possible to determine, without undue experimentation, if a mutant gp120 molecule of the invention elicits a neutralizing human monoclonal antibody response similar to has the same (i.e., equivalent) ability to elicit a response including antibodies having specificity as a human monoclonal antibody such as b12 by ascertaining whether the former or test antibodies prevent the b12 antibody from binding to HIV. If the neutralizing human monoclonal antibody being tested competes with a b12 or b12-like human monoclonal antibody, as shown by a decrease in binding by the non-b12 human monoclonal antibody in standard competition assays for binding to a solid phase antigen, for example, to HIV-1 gp120, then it is likely that the two monoclonal antibodies bind to the same, or a closely related, epitope. In that case, the mutant gp120 molecule utilized to elicit the test monoclonal antibody is a candidate immunogen for eliciting a neutralizing monoclonal antibody response.

[0083] The ability to neutralize HIV at one or more stages of virus infection is a desirable quality of an elicited human monoclonal antibody described herein. Virus neutralization can be measured by a variety of in vitro and in vivo methodologies. Exemplary methods described herein for determining the capacity for neutralization are the in vitro assays that measure inhibition of HIV-induced syncytia formation, plaque assays

and assays that measure the inhibition of output of core p24 antigen from a cell infected with HIV (see also the Examples section below).

[0084] A number of previous mutagenesis studies have reported on the effects of amino acid substitutions on binding by anti-CD4bs antibodies (49, 71, 130, 133, 135). However, only the study by Roben et al. (110) describes such an analysis applied to mAb b12. In that study, wild-type and mutant gp120 were tested for their ability to bind a saturating concentration of each antibody, the results being expressed as the ratio of antibody bound to mutant gp120 and wild-type gp120. This approach is most satisfactory for substitutions that produce large effects in binding, but may be less reliable for detecting smaller effects, since a change in saturation does not necessarily indicate a change in antibody affinity.

[0085] The invention provides methods and compositions for stimulating high levels of broadly neutralizing antibodies. As disclosed herein, the immunospecificity of a human monoclonal antibody of this invention can be directed to epitopes that are shared across serotypes and/or strains of HIV, or can be specific for a single strain of HIV, depending upon the epitope. Thus, a preferred human monoclonal antibody can immunoreact with HIV-1, HIV-2, or both, and can immunoreact with one or more of the HIV-1 strains IIIB, MN, RF, SF-2, Z2, Z6, CDC4, ELI and the like strains. In addition, a preferred human monoclonal antibody can immunoreact and neutralize a majority of field isolates of HIV, as described further herein.

[0086] The immunospecificity of an antibody, its HIV-neutralizing capacity, and the attendant affinity the antibody exhibits for the epitope, are defined by the epitope with which the antibody immunoreacts. The epitope specificity is defined at least in part by the amino acid residue sequence of the variable region of the heavy chain of the immunoglobulin the antibody, and in part by the light chain variable region amino acid residue sequence. Preferred human monoclonal antibodies immunoreact with the CD4 binding site of HIV-1 gp120.

[0087] Also disclosed are immunogenic mutant HIV-1 gp120 molecules having a specified amino acid sequence, which sequence confers the ability to elicit broadly

neutralizing human monoclonal antibodies that bind a specific unique neutralizing epitope and to neutralize HIV when the virus is bound by these antibodies. Such mutant gp120 molecules include, for example, HIV-1 gp120 polypeptides having a substitution of an amino acid corresponding to Gly-473, Asp-474, Met-475, Arg-476, or a combination thereof, for example, a substitution such as G473A, D474A, M475A, R476A, or a combination thereof, for example, at least two amino acid mutations (e.g., D474A and R476A; or G473A and M475A; or D474A, M475A and R476A; or G473A, D474A, M475A and R476A), as well as mutant gp120 polypeptides having D113A, V127A, D180A, N197A, or S256A substitution, or any combination of the above exemplified substitutions. In one embodiment, one or a combination of mutations modifies the gp120 polypeptide so as to generate a glycosylation site (e.g., an *N*-glycosylation site). Such mutations are exemplified by HIV-1 gp120 comprising H92N and N94T; Q114N and L115T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; as well as combinations of such mutations that generate a glycosylation site (e.g., Q114N, L115T, S143T, E150N, and G152T), any or all of which can further include a H92N, K171N, Y173T, and/or I423N substitution. In another embodiment, the mutant gp120 polypeptide comprises combinations of mutations some of which can introduce a glycosylation site into the gp120 polypeptide and others that do not, for example, an immunogenic mutant HIV-1 gp120 polypeptide comprising two or more of G473A, D474A, M475A, R476A, H92N, Q114N, L115T, S143T, E150N, G152T, K171N, Y173T, and I423N.

[0088] In one aspect, neutralizing antibodies, including neutralizing monoclonal antibodies, elicited by mutant gp120 molecules of the invention exhibit a potent capacity to neutralize HIV, particularly neutralizing monoclonal antibodies elicited by an immunogenic mutant HIV-1 gp120 that are specific for HIV-1. The capacity to neutralize HIV is expressed as a concentration of antibody molecules required to reduce the infectivity titer of a suspension of HIV when assayed in an typical *in vitro* infectivity assay such as is disclosed herein. A monoclonal antibody of this invention has the capacity to reduce HIV infectivity titer in an *in vitro* virus infectivity assay by 50% at a concentration of less than

about 700 nanograms (ng) of antibody per milliliter (ml) of culture medium in the assay, and preferably reduces infectivity titers 50% at a concentration of less than about 300 ng/ml, and more preferably at concentrations less than about 10 ng/ml.

[0089] Exemplary monoclonal antibodies described herein are effective at 3-700 ng/ml, and therefore are particularly well suited for inhibiting HIV *in vitro* and *in vivo*.

Particularly preferred human monoclonal antibodies of this invention immunoreact with gp120 in its "mature" form, which form is to be distinguished from antigenic determinants present on the HIV envelope precursor glycoprotein designated gp160. gp160 is processed during virus biogenesis by cleavage into two polypeptides, gp41 and gp120. "Mature" gp120 refers to the processed protein that is found in mature HIV virus particles, and can be detected on the surface of HIV-infected cells.

[0090] Thus, a preferred antibody of this invention binds mature gp120 preferentially over HIV precursor glycoprotein gp160. By "binds preferentially" is meant that the antibody immunoreacts with (binds) substantially more mature gp120 than gp160 in an immunoreaction admixture. Substantially more typically indicates that at least greater than 50% of the total mass of immunoprecipitated material is gp120, and preferably indicates that at least greater than 75%, more preferably 90%, of the immunoprecipitated material is gp120.

[0091] Methods for determining immunoreaction of a subject antibody with gp120 are well known in the art, and the invention need not be so limited. However, preferred methods for determining the relative amounts of envelope glycoprotein antigens include radio-immunoprecipitation (RIP) of cell-surface labeled HIV-infected cells, followed by molecular weight analysis of the labeled products by polyacrylamide gel electrophoresis (PAGE).

[0092] A particularly useful mutant HIV-1 gp120 molecule of the invention can elicit neutralizing human monoclonal antibodies that have the capacity to neutralize a majority of field isolates. As is well understood, the field (i.e., clinically isolated) strains of HIV-1 are typically different to some degree antigenically from laboratory strains. Therefore, it is well

understood that useful neutralizing antibodies must immunoreact with, and be neutralizing against, field isolates of HIV. Preferably, the useful antibody neutralized a large percentage of field isolates, thereby increasing its effectiveness when new strains are encountered.

[0093] U.S. Patent No. 5,652,138 and 5,804,440 demonstrate that the human monoclonal antibody b12 has the ability to neutralize a majority of the field isolates tested. By "majority" is meant that in a representative and diverse collection of field isolates, the antibody is capable of neutralizing at least more than 50% of the strains, and particularly at least about 75% of the strains tested. In this context, "neutralizing" means an effect of reducing the HIV infectivity titre in an *in vitro* virus infectivity assay as described herein at the antibody concentrations described. A preferred antibody is an antibody having the binding specificity of the b12 monoclonal antibody described herein. Preferred are human antibodies having the binding specificity of the immunoglobulin heavy and light chain polypeptides produced by ATCC 69079.

[0094] The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies having the substituted polypeptide also neutralize HIV. By analogy, another embodiment of the invention relates to polynucleotides that encode the above noted heavy and/or light chain polypeptides and to polynucleotide sequences that are complementary to these polynucleotide sequences. Complementary polynucleotide sequences include those sequences which hybridize to the polynucleotide sequences of the invention under stringent hybridization conditions.

[0095] By using a neutralizing human monoclonal antibody of the invention, it is now possible to produce anti-idiotypic antibodies that can be used to screen human monoclonal

antibodies to identify whether the antibody has the same binding specificity as a human monoclonal antibody of the invention and also used for active immunization (Herlyn et al., Science, 232:100 (1986), which is incorporated herein by reference). Such anti-idiotypic antibodies can be produced using well-known hybridoma techniques (Kohler et al., Nature, 256:495 (1975), which is incorporated herein by reference). An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the human monoclonal antibody produced by the cell line of interest. These determinants are located in the hypervariable region of the antibody. It is this region which binds to a given epitope and, thus, is responsible for the specificity of the antibody. An anti-idiotypic antibody can be prepared by immunizing an animal with the monoclonal antibody of interest. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody and produce an antibody to these idiotypic determinants. By using the anti-idiotypic antibodies of the immunized animal, which are specific for the human monoclonal antibody of the invention produced by a cell line which was used to immunize the second animal, other clones with the same idio type as the antibody of the hybridoma used for immunization can be identified. Idiotypic identity between human monoclonal antibodies of two cell lines demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using anti-idiotypic antibodies, other hybridomas expressing monoclonal antibodies having the same epitopic specificity can be identified.

[0096] Anti-idiotypic technology also can be used to produce monoclonal antibodies that mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody. Thus, the anti-idiotypic monoclonal antibody can be used for immunization, since the anti-idiotypic monoclonal antibody binding domain effectively acts as an antigen.

[0097] In one embodiment, the invention contemplates a truncated immunoglobulin molecule comprising a Fab fragment derived from a human monoclonal antibody of this invention. The Fab fragment, lacking Fc receptor, is soluble, and affords therapeutic

advantages in serum half life, and diagnostic advantages in modes of using the soluble Fab fragment. The preparation of a soluble Fab fragment is generally known in the immunological arts and can be accomplished by a variety of methods. A preferred method of producing a soluble Fab fragment is described herein.

[0098] In another embodiment, the invention contemplates an immunoglobulin molecule comprising a Fab fragment derived from a human monoclonal antibody of this invention and the fragment crystallizable (Fc) domain of a human immunoglobulin molecule. The entire (i.e., complete) immunoglobulin (Ig) molecule comprising a Fab fragment with the Fc domain may afford therapeutic and diagnostic advantages, and can be any of the several Ig species depending upon the ultimate use, including IgG, IgA, IgD, IgE, IgM, and isotypes thereof. The immunoglobulin molecule would be capable of effector functions associated with the Fc domain when used in passive immunotherapy. These effector functions include antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cellular cytotoxicity (CDCC) which promote the death of the cell to which the immunoglobulin molecule is specifically bound. The effector functions may therefore be desirable in therapeutic applications. Diagnostic assays include the ability to detect the presence of the immunoglobulin molecule. These assays rely on the cross-linking of red cells or beads in agglutinations, the activation of complement in plaque assays, or the antigenic properties of the Fc region of the heavy chain as detected by secondary antibodies in ELISA or RIA procedures to detect the presence of the immunoglobulin molecule. Such diagnostic assays can only be performed with the entire immunoglobulin molecule. The isolation of the immunoglobulin molecule is also facilitated by the presence of the Fc domain in that commonly used methods of immunoglobulin purification are based upon interaction of reagents with the Fc domain. The preparation of a Fab fragment with the Fc domain is generally known in the immunological arts and can be accomplished by a variety of methods. A preferred method of producing a Fab fragment with the Fc domain is described herein.

[0099] A mutant gp120 polypeptide engineered to elicit neutralizing human monoclonal antibodies can also be used immunotherapeutically for HIV disease. The term

"immunotherapeutically" or "immunotherapy" as used herein in conjunction with an immunogenic mutant gp120 polypeptide such as a mutant HIV-1 gp120 polypeptide of the invention denotes both prophylactic as well as therapeutic administration. Thus, the gp120 mutants can be administered to high-risk patients in order to lessen the likelihood and/or severity of HIV-induced disease, administered to patients already evidencing active HIV infection, or administered to patients at risk of HIV infection.

[0100] The present invention therefore contemplates therapeutic compositions useful for practicing the therapeutic methods described herein. Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with at least one species of gp120 mutant or neutralizing antibodies as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic for non-neutralizing antibodies when administered to a human patient for therapeutic purposes.

[0101] As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a human without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like. The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as sterile injectables either as liquid solutions or suspensions, aqueous or non-aqueous, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

[0102] The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or

emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

[0103] The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

[0104] Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, propylene glycol, polyethylene glycol and other solutes. Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, organic esters such as ethyl oleate, and water-oil emulsions.

[0105] A therapeutic composition contains an HIV-neutralizing amount of a human monoclonal antibody of the present invention or a mutant gp120 molecule of the invention, typically an amount of at least 0.1 weight percent of antibody per weight of total therapeutic composition. A weight percent is a ratio by weight of antibody to total composition. Thus, for example, 0.1 weight percent is 0.1 grams of antibody per 100 grams of total composition.

[0106] For *in vivo* modalities, the method comprises administering to a patient a therapeutically effective amount of a physiologically tolerable composition containing a human monoclonal antibody or mutant gp120 of the invention. Thus, the present invention

describes in one embodiment a method for providing passive immunotherapy to HIV disease in a human comprising administering to the human an immunotherapeutically effective amount of the monoclonal antibody of this invention.

[0107] A representative patient for practicing the present passive immunotherapeutic methods is any human exhibiting symptoms of HIV-induced disease, including AIDS or related conditions believed to be caused by HIV infection, particularly HIV-1 infection, and humans at risk of HIV infection. Patients at risk of infection by HIV include babies of HIV-infected pregnant mothers, recipients of transfusions known to contain HIV, users of HIV contaminated needles, individuals who have participated in high risk sexual activities with known HIV-infected individuals, and the like risk situations. In one embodiment, the passive immunization method comprises administering a composition comprising more than one species of human monoclonal antibody of this invention, preferably directed to non-competing epitopes or directed to distinct serotypes or strains of HIV, particularly HIV-1, so as to afford increased effectiveness of the passive immunotherapy.

[0108] A therapeutically (immunotherapeutically) effective amount of a human monoclonal antibody is a predetermined amount calculated to achieve the desired effect, i.e., to neutralize the HIV present in the sample or in the patient, and thereby decrease the amount of detectable HIV in the sample or patient. In the case of *in vivo* therapies, an effective amount can be measured by improvements in one or more symptoms associated with HIV-induced disease occurring in the patient, or by serological decreases in HIV antigens.

[0109] Thus, the dosage ranges for the administration of the monoclonal antibodies of the invention are those large enough to produce the desired effect in which the symptoms of the HIV disease are ameliorated or the likelihood of infection decreased. The dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art.

[0110] The dosage can be adjusted by the individual physician in the event of any complication. A therapeutically effective amount of an antibody of this invention is typically an amount of antibody such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.1 microgram (îg) per milliliter (ml) to about 100 îg/ml, preferably from about 1 îg/ml to about 5 îg/ml, and usually about 5 îg/ml. Stated differently, the dosage can vary from about 0.1 mg/kg to about 300 mg/kg, preferably from about 0.2 mg/kg to about 200 mg/kg, most preferably from about 0.5 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or several days.

[0111] The human monoclonal antibodies of the invention, mutant gp120 or combination thereof, can be administered parenterally by injection or by gradual infusion over time. Although the HIV infection is typically systemic and therefore most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated where there is a likelihood that the tissue targeted contains infectious HIV. Thus, human monoclonal antibodies of the invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, and can be delivered by peristaltic means.

[0112] The therapeutic compositions containing a mutant gp120 or human monoclonal antibody of this invention are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

[0113] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient

required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for in vivo therapies are contemplated.

[0114] The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the human monoclonal antibodies of the invention, the medicament being used for immunotherapy of HIV disease.

[0115] In a related diagnostic embodiment, the invention contemplates screening HIV-infected patients for the presence of circulating anti-HIV antibodies immunoreactive with gp120 that have a similar epitope immunospecificity when compared to a neutralizing antibody of this invention. Such a screening method indicates that the HIV-infected patient is exhibiting a significant immune response to the virus, and provides useful information regarding disease status and prognosis. The presence of anti-HIV antibodies cross-reactive with a neutralizing antibody of this invention indicates that the patient has some degree of HIV neutralizing activity, as defined herein.

[0116] The diagnostic assay involves determining whether the patient contains human anti-HIV antibodies immunoreactive with the same, similar or overlapping epitopes as a neutralizing antibody of the invention, such that there is a likelihood that there is a useful neutralizing immune response in the patient. There are a variety of immunological assay formats that can be utilized to determine cross-reactivity of test and control antibodies, and the invention need not be so limiting. Particularly preferred are competition assays for a common antigen, preferably in the solid phase.

[0117] The present invention also describes a diagnostic system, preferably in kit form, for assaying for the presence of HIV or an anti-HIV antibody in a sample according to the diagnostic methods described herein. A diagnostic system includes, in an amount sufficient

to perform at least one assay, a subject human monoclonal antibody, as a separately packaged reagent. In another embodiment, a diagnostic system is contemplated for assaying for the presence of an anti-HIV monoclonal antibody in a body fluid sample such as for monitoring the fate of therapeutically administered antibody. The system includes, in an amount sufficient for at least one assay, a subject antibody as a control reagent, and preferably a preselected amount of HIV antigen, each as separately packaged immunochemical reagents. "Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like.

[0118] The present invention provides methods for producing novel HIV-neutralizing human monoclonal antibodies. The methods are based generally on the use of combinatorial libraries of antibody molecules which can be produced from a variety of sources, and include naive libraries, modified libraries, and libraries produced directly from human donors exhibiting an HIV-specific immune response. The combinatorial library production and manipulation methods have been extensively described in the literature, and will not be reviewed in detail herein, except for those features required to make and use unique embodiments of the present invention. However, the methods generally involve the use of a filamentous phage (phagemid) surface expression vector system for cloning and expressing antibody species of the library. Various phagemid cloning systems to produce combinatorial libraries have been described by others. See, for example the preparation of combinatorial antibody libraries on phagemids as described by Kang et al., *Proc. Natl. Acad. Sci., USA*, 88:4363-4366 (1991); Barbas et al., *Proc. Natl. Acad. Sci., USA*, 88:7978-7982 (1991); Zebedee et al., *Proc. Natl. Acad. Sci., USA*, 89:3175-3179 (1992); Kang et al., *Proc. Natl. Acad. Sci., USA*, 88:11120-11123 (1991); Barbas et al., *Proc. Natl. Acad. Sci., USA*, 89:4457-4461 (1992); and Gram et al., *Proc. Natl. Acad. Sci., USA*, 89:3576-3580 (1992), which references are hereby incorporated by reference.

[0119] A method for producing a human monoclonal antibody generally involves (1) preparing separate H and L chain-encoding gene libraries in cloning vectors using

human immunoglobulin genes as a source for the libraries, (2) combining the H and L chain encoding gene libraries into a single dicistronic expression vector capable of expressing and assembling a heterodimeric antibody molecule, (3) expressing the assembled heterodimeric antibody molecule on the surface of a filamentous phage particle, (4) isolating the surface-expressed phage particle using immunoaffinity techniques such as panning of phage particles against a preselected antigen, thereby isolating one or more species of phagemid containing particular H and L chain-encoding genes and antibody molecules that immunoreact with the preselected antigen. As disclosed herein, the resulting phagemid library can be manipulated to increase and/or alter the immunospecificities of the monoclonal antibodies of the library to produce and subsequently identify additional, desirable, human monoclonal antibodies of the present invention. For example, the H chain and L chain immunoglobulin molecule encoding genes can be randomly mixed (shuffled) to create new HL pairs in an assembled immunoglobulin molecule. Additionally, either or both the H and L chain encoding genes can be mutagenized in the complementarity determining region (CDR) of the variable region of the immunoglobulin polypeptide, and subsequently screened for desirable immunoreaction and neutralization capabilities.

[0120] In one embodiment, the H and L chain genes can be cloned into separate, monocistronic expression vectors, referred to as a "binary" system described is further herein. In this method, step (2) above differs in that the combining of H and L chain encoding genes occurs by the co-introduction of the two binary plasmids into a single host cell for expression and assembly of a phagemid having the surface accessible antibody heterodimer molecule.

[0121] In the present methods, the antibody molecules are monoclonal because the cloning methods allow for the preparation of clonally pure species of antibody producing cell lines. In addition, the monoclonal antibodies are human because the H and L chain encoding genes are derived from human immunoglobulin producing immune cells, such as spleen, thymus, bone marrow, and the like.

[0122] The method of producing a HIV-neutralizing human monoclonal antibody also requires that the resulting antibody library, immunoreactive with a preselected HIV antigen, is screened for the presence of antibody species which have the capacity to neutralize HIV in one or more of the assays disclosed herein for determining neutralization capacity. Thus, a preferred library of antibody molecules is first produced which binds to an HIV antigen, preferably gp160, gp120, gp41, the V3 loop region of gp160, or the CD4 binding site of gp120 and gp41, and then is screened for the presence of HIV-neutralizing antibodies as described herein.

[0123] As a further characterization of the present invention the nucleotide and corresponding amino acid residue sequence of the antibody molecule's H or L chain encoding gene is determined by nucleic acid sequencing. The primary amino acid residue sequence information provides essential information regarding the antibody molecule's epitope reactivity.

[0124] As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting between different genetic environments another nucleic acid to which it has been operatively linked. Preferred vectors are those capable of autonomous replication and expression of structural gene products present in the DNA segments to which they are operatively linked. Vectors, therefore, preferably contain the replicons and selectable markers described earlier.

[0125] As used herein with regard to DNA sequences or segments, the phrase "operatively linked" means the sequences or segments have been covalently joined, preferably by conventional phosphodiester bonds, into one strand of DNA, whether in single or double stranded form. The choice of vector to which transcription unit or a cassette of this invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., vector replication and protein expression, and the host cell to be transformed, these being limitations inherent in the art of constructing recombinant DNA molecules.

[0126] The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1

FINE MAPPING OF NEUTRALIZING AND NON-NEUTRALIZING MONOCLONAL ANTIBODY BINDING TO HIV-1 gp120

[0127] This example demonstrates that mutations to gp120 can be used to obtain an immunogenic mutant gp120 polypeptide having increased binding specificity with respect to HIV neutralizing antibodies as compared to non-neutralizing antibodies (see, also, (94)).

[0128] Alanine scanning mutagenesis was performed on monomeric HIV-1 gp120 to systematically identify residues important for gp120 recognition by neutralizing and non-neutralizing monoclonal antibodies (mAbs) to the CD4 binding site (CD4bs). Substitutions that affected binding of broadly neutralizing antibody b12 were compared to substitutions that affected the binding of CD4 and of two non-neutralizing anti-CD4bs antibodies (b3 and b6) with affinities for monomeric gp120 comparable to that of b12. Not surprisingly, the sensitivities to a number of amino acid changes were similar for the mAbs and for CD4. However, in contrast to what was seen for the mAbs, no enhancing mutations were observed for CD4, suggesting that the virus has evolved toward an optimal gp120-CD4 interaction. Although the epitope maps of the mAbs overlapped, a number of key differences between b12 and the other two antibodies were observed. These differences can explain why b12, in contrast to non-neutralizing antibodies, can interact not only with monomeric gp120, but also with functional oligomeric gp120 at the virion surface. Neutralization assays performed with pseudovirions bearing envelopes from a selection of alanine mutants showed a reasonable correlation between the effects of the mutations on b12 binding to monomeric gp120 and neutralization efficacy. However, some mutations produced an effect on b12 neutralization counter to that predicted from gp120 binding data; these mutations appeared to have different effects on the b12 epitope on monomeric gp120 and functional oligomeric gp120. To determine whether monomeric gp120 can be engineered to preferentially bind mAb b12, recombinant gp120 polypeptides were generated containing combinations of alanine substitutions shown to uniquely enhance b12 binding. Whereas b12 binding was maintained or increased, binding by five non-neutralizing anti-CD4bs mAbs (b3, b6, F105, 15e, and F91) was reduced or completely abolished. As

such, the mutant engineered gp120 polypeptides can provide useful immunogens for eliciting broadly neutralizing antibodies.

Materials and Methods

Antibodies

[0129] Monoclonal antibodies (mAbs) b3, b6 and b12 were isolated as Fab fragments from a phage display library derived from a single donor and have been characterized (6, 7, 20, 22, 110). All three antibodies recognize discontinuous epitopes overlapping the CD4bs on HIV-1 gp120.

[0130] CD4-IgG2 is a recombinant antibody-like fusion protein in which the heavy-chain and light-chain variable domains of human IgG2 have been replaced with the D1D2 domains of human CD4 (1). This molecule was used as a surrogate for CD4. MAbs 15e and F91 react with respective epitopes overlapping the CD4bs on gp120 (49, 76, 78, 111), and mAb 17b recognizes an epitope overlapping the coreceptor binding site on gp120 (131, 134, 144).

[0131] Human IgG purified from pooled plasma obtained from healthy asymptomatic seropositive individuals (HIVIG) and mAb F105, another anti-CD4bs antibody (105, 106, 135), were obtained from the NIH AIDS Research and Reference Reagent Program (NIH ARRRP). MAb 2G12 recognizes an epitope involving É1→2 mannose residues on the carbohydrate-rich "silent" face (61, 143) of gp120 (115, 119, 137).

Plasmid constructs and mutagenesis

[0132] Alanine mutations (Table 1, below; Fig. 1A) were generated using the QUIKCHANGE mutagenesis kit (Stratagene). For antibody mapping experiments, plasmid pSVIIIexE7pA_{JR-CSF} was used as the template. This plasmid was derived from pSVIIIexE7pA_{HXB2} (47), which was modified as described previously (154) in order to subclone the *env* gene of HIV-1_{JR-CSF}, a molecularly cloned primary HIV-1 virus (59). Two variable loop-deleted mutants were also generated, using pSVIIIexE7pA_{JR-CSF} as the PCR template. A V1-deleted (ΔV1) mutant (deletion of residues 134-154) was constructed by PCR using the primer pair csf120-f

(5'-GTCTGAGTCGGAGCTAGCGTAGAAAAGTTGTGGGTCA-3'; SEQ ID NO:1), and csfdV1-r (5'-GTCTGAGTCGGA**AACCGG**ACCCATCTTTGCAATTAAAGTA-3'; SEQ ID NO:2); and the primer pair csfdV1-f (5'-GTCTGAGTCGGAT**TCCGGT**TCTGGGAAAACTGCTCTTT-3'; SEQ ID NO:3), and csf120-r (5'-GTCTGAGTCGGACTCGAGTTTCTCTTTGCACCACTCTTC-3'; SEQ ID NO:4). Primers csfdV1-f and csfdV1-r both contain a *BsaW* I restriction site (bold).

[0133] The PCR products were cloned into pSVIIIexE7pA⁻_{JR-CSF} using *Kpn*I, *BsaW* I and *Mfe*I in a 2-step ligation reaction. A γ V3-mutant (deletion of residues 303-324) was generated in a similar manner using the primers csf120-f (SEQ ID NO:1) and csfdV3-r (5'-GTCTGAGTCGGA**AACCGG**ACCCATTGTTGCTGGGCCTTGT-3'; SEQ ID NO:5), and the primers csfdV3-f (5'-GTCTGAGTCGGAT**TCCGGT**TCTGGGGATATAAGACAAGCCC -3'; SEQ ID NO:6) and csf120-r (SEQ ID NO:4). Primers csfdV3-f and csfdV3-r also contain unique *BsaW* I restriction sites.

[0134] To generate a γ V1/V2-mutant (V2 loop deleted from residues 160-193), the pSVIIIexE7pA⁻_{JR-CSF}, V1 mutant was used as a template. First, the *BsaW* I site introduced into the γ V1-mutant was changed by site-directed mutagenesis, whereby the amino acid sequence was retained. Deletion of the V2 sequences was performed in an analogous manner as for the γ V1- and γ V3-mutants, using the primers csf120-f (SEQ ID NO:1) and csfdV2-r2 (5'-GTCTGAGTCGGA**AACCGG**ACCCGAAAGAGCAGTTTTT-3'; SEQ ID NO:7) and the primers csfdV2-f (5'-GTCTGAGTCGGAT**TCCGGT**TCTGGGATAAGTTGTAACACC-3'; SEQ ID NO:8) and csf120-r (SEQ ID NO:4). The unique *BsaW* I restriction sites in primers csfdV2-r2 and csfdV2-f are shown in bold. PCR fragments were cloned into pSVIIIexE7pA⁻_{JR-CSF} using *Kpn*I, *BsaW* I and *Mfe*I. In all variable loop-deleted mutants, the deleted sequences were replaced by a Gly-Ser-Gly-Ser-Gly linker.

[0135] Mutants containing multiple alanine substitutions were also generated using the QUIKCHANGE mutagenesis kit, except that the plasmid pCMV-Tag-tpa_{JR-FL}gp120 was used as the template. This plasmid was derived from plasmid pCMV-Tag4A (Stratagene). First, a DNA fragment encoding the tissue plasminogen activator protein (Tpa) was generated by PCR using the primers tpa1

(5'-CGTT**GAATTC**GCCTCCACCATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCG-3'; SEQ ID NO:9), which contains an *EcoR* I restriction site (bold) and tpa2

(5'-GCACCT**CGAGGCGGCT**CCTCTTCTGAATCGGGCATGGATTTCCTGGCTGGGCGAAACGAAGACTGCTCCACACAG-3'; SEQ ID NO:10), which contains *XhoI* and *BssH* II restriction sites (bold and bold/italics, respectively). This fragment was cloned into pCMV-Tag4A using *EcoR* I and *XhoI*, to generate pCMV-Tag-tpa. The *env* gene from HIV-1_{JR-FL} (59) was amplified by PCR using as template the plasmid pSyngp140_{JR-FL} (obtained from the NIH ARRRP; 2, 43(2)), in which most wild-type gp140 codons were replaced with codons from highly expressed human genes. For PCR, the primers jr-fl5' (5'-CGTT**GCGCGC**GTGGAGAAGCTGTGGGTG-3'; SEQ ID NO:11), which contains a *BssH* II restriction site (bold), and

flXho-r (5'-GCAGAGGGAGAAGCGCCT**CGAGG**CTGTGGGCATTGG-3'; SEQ ID NO:12), which contains a *XhoI* restriction site (bold), were used. The PCR fragment was cloned into pCMVtag-tpa using *BssH* II and *XhoI*, to generate pCMVtag-tpa_{JR-FL}gp120. All plasmids and mutations generated in this study were verified by DNA sequencing.

Generation of recombinant HIV-1 virions

[0136] To produce recombinant virions, 293T cells grown in Dulbecco's modified Eagle's media (Gibco) supplemented with penicillin, streptomycin, L-glutamine and fetal bovine serum (10%) were transiently transfected with wild-type or mutant pSVIIIexE7pA⁻_{JR-CSF} plasmids (2 µg) along with the luciferase reporter plasmid pNL4-3.Luc.R'E⁻ (4 µg, obtained from the NIH ARRRP (26, 46)) using FuGENE6 transfection reagent (Roche). At 24 hr post-transfection, the culture supernatant was replaced with serum-free media and incubation was continued for another 24 hr. Cell culture supernatants, containing

pseudovirions, were subsequently harvested and stored at -80°C for neutralization assays (see below). Alternatively, recombinant virions were lysed by the addition of detergent to the harvested culture supernatant, which was then stored at -20°C until further use.

Expression of recombinant gp120

[0137] pCMVtag-tpa_{JR-FL}gp120 plasmids expressing wild-type or mutant gp120 were used to transiently transfect subconfluent 293T cells grown in serum-containing media as described above, except that no pNL4.3Luc was used. Two days post-transfection, culture supernatants containing recombinant gp120 proteins were collected and stored at -20°C.

ELISA assays

[0138] For ELISA, microtiter plate wells (flat bottom, Costar type 3690, Corning Inc.) were coated overnight at 4°C with anti-gp120 antibody D7324 (International Enzymes, Inc.) at a concentration of 5 μ g/ml (250 ng/well, diluted in PBS). Subsequent incubation steps were performed at room temperature. Coated plates were washed twice with PBS supplemented with 0.05% Tween (PBS-T), blocked for 1 hr with PBS supplemented with 3% BSA and subsequently incubated for 2-4 hr with cell culture supernatant that was diluted 1:3 in PBS containing 1% BSA and 0.02% TWEEN-20 detergent (PBS-B-T). Plates were washed with PBS-T (10 times), then incubated with mAb serially diluted in PBS-B-T (starting at a concentration of 10 μ g/ml). HIVIG (1 μ g/ml, diluted in PBS-B-T) was used as a control to ensure that similar amounts of envelope protein were captured. After washing as before, peroxidase-conjugated or alkaline phosphatase-conjugated goat anti-human IgG (F(ab')₂-specific, Pierce) was added (diluted 1:1000 in PBS-B-T) and incubation continued for another hour. Plates were washed again, followed by incubation with TMB-substrate (Pierce) when peroxidase-conjugated secondary antibody was used or *p*-nitrophenyl phosphate (Sigma) when alkaline phosphatase-conjugated secondary antibody was used. The color reaction was stopped by adding 2 M sulfuric acid (in the case of TMB) and absorbances were measured at 450 nm. Assays developed with *p*-nitrophenyl phosphate were measured at 405 nm without stopping the reaction. Apparent affinities were calculated as the antibody concentration at half-maximal binding; percent changes in affinity relative

to wild type were expressed as (apparent affinity of the wild type/apparent affinity of the mutant) x 100.

HIV-1 neutralization assays

[0139] Recombinant virions competent for a single round of infection were generated as described above. Neutralization assays were performed essentially as described previously (154), using an initial seeding density of between $1-3 \times 10^4$ target cells (U87.CD4.CCR5, obtained from the NIH ARRRP). The degree of virus neutralization by antibody was determined by measuring the luciferase activity.

[0140] The percent neutralization at a given antibody concentration was expressed as $\{(\text{luciferase activity in the absence of antibody} - \text{luciferase activity in the presence of a given antibody concentration}) / \text{luciferase activity in the absence of antibody}\} \times 100$. To determine the degree of correlation between neutralization efficiency and the change in antibody binding affinity for each mutant relative to wild type, a neutralization index was defined. This index was expressed as $\{(\text{antibody concentration required to achieve 90\% neutralization of the wild type} \times \text{apparent antibody affinity for wild-type-gp120}) / (\text{antibody concentration required to achieve 90\% neutralization of the mutant} \times \text{apparent antibody affinity for mutant gp120})\}$. Neutralization indexes of between 0.2 and 5 were considered indicative of a reasonable correlation between the change in antibody affinity for monomeric gp120 and neutralization efficiency.

RESULTS

[0141] Alanine mutagenesis was performed on monomeric gp120 to define in more detail which residues on gp120 influence or modulate b12 reactivity. In parallel, CD4-IgG2 (used as a surrogate for CD4) and two non-neutralizing anti-CD4bs mAbs (b3 and b6 (6)) were assayed to compare the effects of alanine mutations on binding by neutralizing versus non-neutralizing mAbs, as well as to distinguish between amino acid substitutions that uniquely affect mAb binding and mutations that affect the binding of anti-CD4bs ligands in general. Mutagenesis was performed using gp120 from HIV-1_{JR-CSF} as the parent. A total of 81 mutants containing single alanine substitutions and 3 variable loop-deleted mutants

were generated (Fig. 1A). The residues that were mutated to alanine were selected primarily from a list of likely contact residues based on the docking model of the crystal structure of mAb b12 and the CD4-complexed gp120 core structure of HxB2 (90). Most mutations were in or adjacent to the CD4bs (Fig. 1B), encompassing the neutralizing as well as the non-neutralizing faces of gp120 (Fig. 1C). However, a number of amino acids that were selected for mutagenesis were also located on the silent face. As shown in Fig. 1D, amino acids that were selected for mutagenesis ranged from those that are highly conserved among primate immunodeficiency viruses to residues that vary significantly among HIV-1 isolates.

[0142] To determine apparent antibody affinities, mutant monomeric gp120 from pseudovirions was captured onto ELISA plate wells and probed with varying concentrations of antibody to generate a binding curve for each mutant. Apparent binding affinities were determined from the antibody concentration at half-maximal binding. The apparent antibody affinity for each mutant gp120 was then related to that for wild-type gp120 (Table 1, below). Changes in relative affinity greater than 200% were designated as increases, whereas those below 50% were designated as decreases. Intermediate values were recorded as having no or limited effect on antibody binding.

[0143] Three variable loop-deleted gp120 mutants (ΔV1, ΔV1/V2 and ΔV3) were investigated. Deletion of the V1 loop alone or together with the V2 loop had an adverse effect on the binding of CD4 and all three mAbs to gp120, whereas deletion of the V3 loop decreased the binding affinity of CD4 and mAbs b6 and b12, but not b3 (Table 1, below). Nineteen alanine substitutions in gp120 reduced the affinity for CD4 and all three mAbs. Three substitutions (at D180, I184, F176) are located in the V2 loop, one (at K207) is located at the base of the V1/V2 loop-stem, one (at I213) is located on the non-neutralizing face at the putative gp120-gp41 interface (61, 143), six (at T257, E370, I371, Y384, M426, G472) line, i.e., the Phe43 cavity (61) of gp120, four (at N386, P470, R476, W479) are in close proximity to the CD4 binding pocket (61), three (at R350, W395, T450) are on the carbohydrate-rich silent face of gp120 and one (at I439) is close to the junction of silent and neutralizing faces (61, 143), adjacent to the base of the stem of the V1/V2 loop. Except for

R350 and W395, these residues are all conserved among primate immunodeficiency viruses or HIV-1 isolates in terms of identity or similarity of the amino acid side chain (Table 1, below). Four of the nineteen residues listed above (E370, I371, M426 and G472) are CD4 contact residues (Table 1, below; (61)), and their conservation is probably required for the optimal interaction of gp120 with CD4. For the remaining residues, conservation may be associated with maintenance of the structural integrity of gp120 (61, 143) and it is possible that substitution by alanine leads to local misfolding of monomeric gp120.

[0144] To determine whether monomeric gp120 from these mutants was globally perturbed, the binding of mAb 2G12, which recognizes a carbohydrate-dependent conformational epitope on the silent face of gp120 (115, 119, 137), also was investigated. For most mutant glycoproteins, 2G12 binding was unchanged (Table 1, below). It would thus appear that these proteins are not globally misfolded. Interestingly, mutating residue F176 (V2 loop) to alanine caused a fourfold increase in 2G12 binding relative to that seen with wild-type gp120, indicating that mutations in the V2 loop of gp120 can have some effect on 2G12 binding to its carbohydrate epitope. Alanine substitutions of three residues (N386, W395, and W479) caused moderate to significant decreases in 2G12 relative affinity (Table 1, below). None of these residues is believed to be a contact residue for mAb 2G12 (115, 119, 137), suggesting that these alanine replacements may cause significant perturbations of the gp120 structure.

[0145] The decrease in CD4 binding affinity observed with for the W395A mutant was somewhat striking, considering that this residue shows significant variability among HIV-1 isolates (61). However, alignment of the sequences from HIV-1 clade B isolates showed that this amino acid is identical in 106 of the 107 isolates listed in the HIV sequence database (see hypertext transfer protocol ([http](http://hiv-web.lanl.gov/content/hiv-dp/mainpage.html)), at URL hiv-web.lanl.gov/content/hiv-dp/mainpage.html). In the single clade B isolate in which this amino acid is not Trp, it is replaced by a His residue. Position 395 may thus play a role in preserving a structural conformation that is required for optimal CD4 binding in clade B isolates and that is achieved by the incorporation of aromatic amino acids. The same may hold true for R350,

which is 60% conserved as arginine in clade B isolates but 80% conserved as a positively charged side chain.

[0146] The remaining 62 alanine substitutions had varying effects on CD4- and antibody-reactivity (Fig. 2). A noticeable difference between the effects of alanine substitution on ligand binding was that, whereas some substitutions enhanced mAb binding, CD4 binding was always either decreased or unchanged. Many substitutions produced similar effects on mAb and CD4 binding (Table 1, below; Fig. 2). For 31 mutations, the effects on b3 binding were similar to the effects on CD4 binding. In comparison, the effects on b6 and b12 binding were similar to the effect on CD4 binding for 24 and 26 mutations, respectively. However, if a focus was directed on substitutions that decreased ligand binding, then the greatest correspondence was between mAb b12 and CD4, closely followed by mAb b6 and CD4, and with the greatest discrepancy being between mAb b3 and CD4. Thus, excluding the previous 19 alanine substitutions which uniformly reduced binding by CD4 and the three mAbs, 18 substitutions decreased binding of both b12 and CD4, 17 decreased binding for both b6 and CD4, and only 11 decreased binding for CD4 and for b3.

[0147] Amino acid substitutions that affected mAb binding were mapped onto the crystal structure of the gp120 core of HIV-1_{HXB2}, to obtain a better understanding of the spatial arrangement among the amino acid residues that were mutated (Fig. 3). Although the mutagenesis studies used gp120_{JR-CSF}, this approach was thought to be valid since the structure of the core seems to be highly conserved among HIV-1 isolates (60). One caveat to note is that the structure of gp120 is that of a core molecule complexed to CD4 and Fab 17b; some differences in the conformation of this molecule and the corresponding unliganded molecule have been proposed (61, 143, 145), but no structural data to address this issue yet exists. The epitope maps for the antibodies, particularly mAbs b6 and b12, were highly similar (Fig. 3). This is not surprising, given that anti-CD4bs antibodies compete with each other for binding to gp120 (80). The map observed for CD4 was in good agreement with the CD4 footprint derived from the crystal structure of the CD4-gp120 complex (61, 143). Notably, the map for b12 was also in agreement with the footprint obtained from a docking model of the b12 structure and the gp120 core structure (90).

[0148] To gain a better understanding of the differences between each mAb, only those alanine substitutions for which the effect (or lack of effect) on antibody reactivity was unique compared to the other two antibodies were mapped onto the gp120_{HXB2} core structure (Fig. 4, top panel). The differential map obtained for mAb b3 shows a cluster of several residues (G366A, G367A, T388A, K421A, D457A, G458A and R469A) on the neutralizing face (61, 143) and close to the V1/V2 loop stem (61, 143) of gp120 (K121A, V430A) that, when mutated to alanine, uniquely do not affect b3 binding. This result implied that the epitope recognized by b3 may not involve these particular regions. The differential map for mAb b6 shows that, in contrast to b3 and b12, it is not affected by certain substitutions (D279A and E462A) in the upper region of the neutralizing face of gp120 or by some alanine mutations which cluster around the Phe43 cavity (D368A, W427A, G473A). The differential map for mAb b12, in contrast to the maps of the other two antibodies, shows little clustering of amino acid mutations that uniquely do not affect b12 binding. Rather, alanine substitutions to which b12 is uniquely insensitive are located at the tip of the V1/V2-stem (V127A), at the junction of the neutralizing and non-neutralizing faces (D474A and M475A) and in the upper half of the neutralizing face (I467A). Interestingly, most mutations that uniquely reduce b12 binding were located in close proximity to the Phe43 cavity (Fig. 4, top panel).

[0149] In addition, those alanine substitutions that had a unique effect on b3 or b6 (non-neutralizing antibody) binding compared to b12 (neutralizing antibody) binding were mapped (Fig. 4, bottom panel). The differential map for b3 showed that it was not affected by many alanine substitutions, particularly those which are on the perimeter of the CD4bs, as compared to b12. The strikingly high number of colored residues in the map for b3 suggests that b3 and b12 differ significantly in gp120 contact residues. Indeed, many of the residues that, when substituted by alanine, uniquely caused a decrease in b3 binding as compared to b12 are located on the non-neutralizing face and inside face of the bridging sheet which faces the non-neutralizing face. The differential map for b6 showed fewer colored residues as compared to the map obtained for b3, which suggests that there are few differences between b6 and b12 in their interaction with gp120.

[0150] Mutants which are able to escape neutralization by mAb b12 have been generated *in vitro* and *in vivo* (74, 104) and are characterized by amino acid mutations in the V2 loop (D167N, D185N), as well as in the C3 region (P369L, P369Q), adjacent to the Phe43 cavity. To determine the extent to which alanine substitutions in the current study resulted in neutralization escape, 19 alanine mutants with varying effects on b12 binding affinity for monomeric gp120 were selected to encompass the entire gp120 envelope and used in an assay in which an *env*-defective HIV-1 provirus encoding the firefly luciferase gene (pNL4.3Luc) is complemented for a single round of infection by a plasmid encoding wild-type or mutant envelope glycoproteins (Table 2, below). A neutralization index was defined to determine the degree of correlation between antibody affinity for monomeric gp120 and neutralization efficiency.

[0151] For 12 mutants (K97A, T123A, L125A, R166A, F210A, R252A, T283A, Q337A, P369A, G459A, G471A and D474A), there was a reasonable correlation between neutralization sensitivity of pseudovirions expressing mutant gp120 and antibody affinity for monomeric gp120 of the corresponding mutant. For the remaining seven alanine mutants, discrepancies were observed between the change in b12 binding affinity for monomeric gp120 and neutralization efficiency (Table 2, below; Fig. 5). Strikingly, for five mutants (D113A, V127A, D180A, N197A and S256A), neutralization efficiency was maintained or increased despite a decrease in antibody affinity for monomeric gp120. For instance, whereas the binding affinity of b12 for monomeric gp120 from the D113A virus was reduced 100-fold compared to wild-type gp120, D113A virus was neutralized as efficiently as wild-type virus by b12. In contrast, the neutralization indices for the remaining two mutants, N276A and S365A, were low (0.14 and 0.04, respectively), because of decreased neutralization efficiency despite an increase in binding affinity for monomeric gp120. These results suggest that antibody affinity changes observed with amino acid substitutions on monomeric gp120 are not always maintained in the context of functional oligomeric gp120 as present on the surface of the virus.

[0152] To determine whether the observed discrepancies between binding to monomeric gp120 and neutralization were only applicable to b12, mAbs CD4-IgG2, 2G12, 15e and 17b

also were tested with six of the seven mutants described above in neutralization assays. Mutant V127A was excluded because the observed neutralization index was only slightly higher than the cut-off value and thus considered only marginally discrepant. MAb 15e recognizes an epitope overlapping the CD4bs on gp120 (111), whereas mAb 17b recognizes an epitope which overlaps the coreceptor binding site on gp120 and is better exposed upon CD4 binding (134). Both mAbs, which neutralize HIV-1 primary isolates poorly (35, 72, 136), were not able to achieve 90% neutralization of any of the mutant viruses or wild-type virus at concentrations up to 100 μ g/ml, although both mAbs bound monomeric gp120 from all mutants with affinities comparable to wild-type gp120. This result suggested that the introduced mutations did not dramatically increase the neutralization sensitivity of JR-CSF to these mAbs. Further, all six mutants remained similarly sensitive to neutralization by mAb 2G12 as the wild-type virus (Table 3, below). Therefore, it is likely that the changes in neutralization efficiency observed with mutant pseudovirions are restricted to the b12 epitope. Interestingly, for mutant S365A, a neutralization index <0.2 for mAb 2G12 was observed; 2G12 had a higher affinity for monomeric gp120 from this mutant, but the corresponding pseudovirions were not neutralized better than wild-type virus. In view of the location of the 2G12 epitope on gp120 (115, 119, 137) and considering that residue S365 is located in the CD4bs of gp120, mutating this residue to alanine appears to cause a conformational change in monomeric gp120 that leads to better presentation of one or more glycans on the silent face of gp120 to which 2G12 binds. However, this conformational change did not appear to take effect on the viral spike, since mutant viruses were not neutralized more efficiently than wild-type viruses.

[0153] For mAb CD4-IgG2, neutralization efficiency was decreased with respect to wild-type virus with two (S256A and N276A) of the six mutant pseudovirions that were tested (Table 3, below). This decrease correlated well with the observed decrease in CD4-IgG2 affinity for monomeric gp120 (Table 3, below). For the remaining 4 mutants, there was a discrepancy between neutralization efficiency and antibody affinity for gp120. For mutants D113A, D180A and N197A, the same discrepancy was observed as with b12 - an increase in neutralization efficiency despite a decrease in binding affinity for monomer.

Interestingly, for mutant S365A, the effects on CD4-IgG2 and b12 binding and neutralization diverged. Monomeric gp120 of mutant S365A showed enhanced affinity for b12 and reduced affinity for CD4-IgG2. However, whereas the corresponding virus was neutralized by CD4-IgG2 equally well as wild type, a decrease in neutralization efficacy was observed for b12 with pseudovirions from mutant S365A. Thus, although mutations in gp120 can affect the binding of b12 and CD4-IgG2 to monomeric gp120 differently, these differences are apparently not necessarily maintained when gp120 is oligomerized.

[0154] Although mAb b12 and non-neutralizing anti-CD4bs antibodies bind monomeric gp120 with similar affinity, it was hypothesized monomeric gp120 could be mutated so that it preferentially binds b12, but not non-neutralizing mAbs. Based on structural analysis (Fig. 4), it was apparent that a region encompassing amino acids G473-R476, which partially line the Phe43 cavity, uniquely affected b12 binding as compared to mAbs b3 and b6 (Fig. 4, right panel). Accordingly, a small panel of recombinant gp120 proteins with multiple alanine substitutions at these amino acid positions was generated to determine whether the unique differences in the effects observed with the single alanine mutations could be retained. For this, a plasmid was constructed encoding the gp120 segment of a codon-optimized *env* gene of the primary isolate JR-FL, which is 94% identical in amino acid sequence to JR-CSF. A tissue plasminogen activator leader sequence was placed upstream of the *env* gene to ensure secretion of gp120 into the culture media. Four mutants (GDMR, DMR, DR and GM) were generated and tested on a panel of anti-CD4bs mAbs (Table 4, below). The results show that, with the double (DR, GM) and triple (DMR) mutants, b12 binding affinity was similar to wild-type gp120, whereas with the quadruple mutant (GDMR), b12 binding was increased. In contrast, the binding affinities of mAbs b3, b6 and F105 were severely reduced with all four mutants. CD4 binding was also severely diminished with the GDMR, DMR and GM mutants, but not with the DR double mutant (twofold reduction in affinity relative to wild type). Two other anti-CD4bs antibodies, mAbs F91 and 15e, were not as susceptible to alanine substitutions in this region as compared to the other mAbs; none of the mutations produced a decrease of more than 50% with either of the two antibodies. The binding affinity of mAb F91 was affected most by

mutant DMR, whereas the binding of mAb 15e was affected most by mutants GDMR and GM. This result suggested that these mAbs might bind to gp120 differently than other anti-CD4bs antibodies. Indeed, the binding of mAb F91 is uniquely enhanced in the presence of anti-V2 and -V3 loop antibodies (80), whereas both mAbs enhance the binding of several anti-V2 and anti-V3 loop mAbs (80). The other anti-CD4bs antibodies tested herein do not generally display similar effects. Binding of mAb b12, for example, is decreased in the presence of a number of anti-V2 loop antibodies (80).

[0155] The CD4bs on gp120 is a particularly attractive target for HIV-1 vaccine design because CD4 is the primary receptor on target cells for virtually all naturally occurring viruses studied to date (30, 54), thus suggesting a common structural framework for the binding site among all HIV-1 isolates. This notion is supported by the limited sequence variability among amino acids which make up the CD4 binding pocket (58, 61, 91), as well as by the high degree of similarity between the crystallized gp120 core structures of a TCLA strain and a primary isolate (60). Of the three broadly neutralizing anti-gp120 mAbs that have been described to date, including b12, 2G12 and Fab X5, only the former binds to an epitope directly overlapping the CD4bs (6, 20, 22, 110).

[0156] As disclosed herein, the amino acid residues of gp120 that affect binding were systematically defined using the broadly neutralizing mAb b12, CD4 and two non-neutralizing anti-CD4bs antibodies. Selected residues were changed to alanine because alanine generally does not significantly alter the main-chain conformation or impose extreme electrostatic or steric effects and so permits identification of amino acid side chains, which may be important for ligand binding. To determine antibody affinity changes, antibody binding curves from ELISA data were generated, and the apparent antibody affinity for each gp120 mutant was determined and related to that for wild-type gp120.

[0157] The effects of alanine substitutions on CD4 binding were determined using CD4-IgG2 because it allowed the same detection system to be used as for the antibodies. No increase in affinity was observed for CD4 with any of the alanine mutants tested (see

Table 1, below; Figures 2 and 3), consistent with a drive towards selection of a particular ensemble of residues during viral evolution for optimal CD4 binding. In fact, of the 28 amino acid substitutions that diminished CD4 binding to less than 20% of wild type (Table 1, below), all but one (I467) were conserved (as defined by amino acid identity or conservation of the amino acid type) among primate immunodeficiency viruses or HIV-1 isolates, and/or are CD4 contact residues. The decrease in CD4 reactivity can reflect the loss of certain functional or structural features required to maintain the integrity of the CD4bs. Twenty-nine mutations caused a moderate decrease (20-50%) in CD4 binding affinity. Of these 29 residues, 21 are conserved among primate immunodeficiency viruses or HIV-1, and/or are CD4 contact residues. The other 8 residues (K171, F210, R252, R273, N276, R350, W395, E462) showed moderate to significant variability among HIV-1 isolates; the affinity changes observed with CD4 when these amino acids were mutated to alanine can relate specifically to the HIV isolate, i.e. JR-CSF.

[0158] Although the results disclosed herein are largely in agreement with those of Olshevsky et al. (91), wherein a panel of gp120 mutants was tested for binding to CD4⁺-target cells, a direct comparison between that study and the present results is difficult due to the different assay formats (Olshevsky et al. used densitometric quantitation of autoradiograms of immunoprecipitated gp120 mutants after incubation of the mutants with CD4⁺-target cells). Minor discrepancies also can be due to differences in amino acid substitutions, wherein Olshevsky et al. substituted by many different amino acids, whereas the present study utilized only alanine.

[0159] As disclosed herein, many amino acid changes affected b3, b6 and b12 binding similarly (Figure 2 and 3), indicating a high degree of overlap in gp120 determinants recognized by these anti-CD4bs antibodies. This result was not surprising, considering that anti-CD4bs antibodies compete with each other for binding (80). Increases in gp120 binding affinity were observed with a number of alanine substitutions, in contrast to CD4. An increase in affinity may indicate that the amino acid in the wild type sterically hinders antibody binding. Typically, alanine substitutions result in a smaller side chain at a given

position that may decrease steric interference and increase antibody affinity. However, most mutations had an adverse effect on their binding ability (Figures 2 and 3).

[0160] Deletion of the V1 and/or V2 loops diminished binding by all three mAbs as well as CD4. Deletion of the V3 loop also diminished binding by CD4 and by mAbs b6 and b12. These results indicate that the variable loops can affect binding of anti-CD4bs antibodies (146). Alanine substitutions in the V2 loop and the C-terminal strand of the V1/V2-stem generally decreased the binding affinity of b12 more severely than of b3 and b6. Previous studies showed that b12 is sensitive to changes in the V1/V2-stem loop structure (12), to deletion of V1/V2 (12) and to mutations in the V2 loop (74, 110). The results obtained here are consistent with the previous observations, although it is noted that mAb b12 does not appear to be uniquely sensitive to V1/V2 deletion. These results are further supportive of the assumption (143) that the V2 loop in particular is in close proximity to the Phe43 cavity on the gp120 core. The mutagenesis results are also consistent with those from previous studies, since many of the substitutions that adversely affect antibody binding (e.g. D113, S256, T257, N262, E370, D368, K421, D477) diminish binding by other anti-CD4bs mAbs (49, 71, 130, 133, 135).

[0161] Unique differences between each antibody were mapped onto the gp120 core structure to gain better insight into how the neutralizing antibody b12 differs from the other non-neutralizing anti-CD4bs antibodies (Figure 4). The large cluster of residues on the neutralizing face of gp120, which uniquely do not affect b3 binding, suggests that the non-neutralizing face and inner domain (143) of gp120, and to a lesser extent the neutralizing face, form a major contact region for the b3 antibody. This is also supported by the insensitivity of mAb b3 to removal of the V3 loop.

[0162] For mAb b6, some substitutions that uniquely affected antibody binding were located close to or facing the non-neutralizing face, suggesting that b6 may interact with an epitope extending across the non-neutralizing and neutralizing faces, but at an angle inclined toward the non-neutralizing face. Based on its unique differences with mAbs b3

and b12, b6 appeared to contact very few residues that line the Phe43 cavity, suggesting that this region is less involved in the b6 epitope.

[0163] For b12, no spatial clustering was observed for residues that uniquely do not affect binding (Fig. 4). Most mutations that uniquely affected b12 binding were located in the neutralizing face. This observation and consideration of the epitope map obtained for b12 indicate that the epitope recognized by b12 is located primarily in this region. This results supports the computational docking model of the gp120 core structure and mAb b12 (90), in which b12 binds to an epitope extending from the stem of the V1/V2 loops across the neutralizing face of gp120, with little contact to the non-neutralizing face. Figure 4 (top panel) also shows that a number of mutations around the Phe43 cavity on gp120 uniquely diminished b12 binding, supporting recent results that residues comprising the antigen binding region, particularly those in the extended finger-like loop of the third complementarity determining region (CDR) of the heavy chain of Fab b12, make crucial contacts with the residues close to the CD4 binding pocket on the gp120 surface (90, 153).

[0164] A model was sought to explain the differences between b12 and non-neutralizing antibodies, based on the data and the epitope maps obtained here. Figure 6 depicts how two hypothetical non-neutralizing antibodies can be excluded from interacting with trimeric gp120, in contrast to b12 which binds effectively. As shown in Figures 6C and 6D, non-neutralizing antibodies binding to gp120 molecules in a functional trimer can be hindered by the close proximity of a neighboring gp120 molecule. In contrast, mAb b12, in this view, is not hindered by adjacent gp120 protomers because the angle of interaction permits binding to both monomeric as well as oligomeric gp120 (Figure 6B).

[0165] The neutralization sensitivity of pseudovirions from a select number of alanine mutants was determined, to investigate how well changes in b12 binding affinity for monomeric gp120 correlated with neutralizing ability. For this, a neutralization index was defined: mutants with high indices are neutralized better or equally well by mAb b12 as compared to wild type virus, despite a decrease in b12 binding affinity for monomeric gp120, whereas mutants with very low indices are neutralized worse than wild type

pseudovirions, despite an increase in b12 binding affinity for monomer. Although there was a reasonable correlation for most mutants, e.g. an increase in b12 binding affinity and an increase in neutralization efficacy, discrepancies were observed for 7 of the 19 mutants that were selected (Table 2). This result indicates that the effects of certain substitutions on antibody binding to monomeric gp120 are nullified or reversed when presented in the context of a functional oligomeric gp120 complex on the viral surface, because the ability of an antibody to bind functional oligomeric gp120 is generally believed to correlate with neutralization efficacy (35, 95, 100, 116). Interestingly, mutants for which there was a discrepancy between binding to monomer and neutralization were located on opposite sides of the core (Figure 5).

[0166] Three of the five mutants (D113A, N197A and V127A) for which b12 binding affinity for monomeric gp120 was reduced, but which were still neutralized efficiently by the antibody (neutralization indices of 233, 56 and 5.65, respectively; Table 2), were located in close proximity to or on the V1/V2 stem on the inside face of the bridging sheet which faces the non-neutralizing face. Considering that the location of the V1/V2 loop may affect b12 binding, the substitutions in the stem region can lead to a repositioning of the V1/V2 loops, the effect of which is markedly different for monomeric and functional oligomeric gp120; i.e., the substitutions lead to increased obstruction of the CD4bs for monomeric gp120, but have little effect for oligomeric gp120. These observations are reminiscent of results obtained recently by Kolchinsky et al. (55, 56), in which mutant pseudovirions of the primary isolate ADA with an N→K or Q substitution at position 197 become highly sensitive to neutralization by various anti-gp120 mAbs (56). These mutations, which eliminate an *N*-linked glycan at position 197, may cause movement of the V2 loop, as inferred from the ability of these viruses to infect target cells independent of CD4 (55, 56). Surprisingly, the N197A mutant was not sensitive to neutralization by mAbs 15e and 17b, which neutralized ADA viruses containing the N197K mutation well (56). The exceptional sensitivity of the ADA mutant pseudovirions to antibody neutralization may relate specifically to ADA, and not apply to other HIV isolates to the same extent. In this respect,

preliminary results indicated that the N197A mutant of JR-CSF was not able to infect target cells that do not express CD4.

[0167] The other two mutants, D180A and S256A, for which a similar discrepancy was observed between binding to monomer and virus neutralization, are located in the V2 loop and line the Phe43 cavity, respectively. It is likely that changing residue D180 to alanine also influences the position of the V2 loop. The reason for the observed discrepancy with mutant S256A is not readily apparent. Residue S256 lies recessed in the Phe43 cavity of the CD4-liganded conformation of gp120, at the interface between the inner and outer domains (61), and, therefore, is unlikely to contact b12 directly. Replacing this residue with alanine may affect the spatial orientation of the inner and outer domains on monomeric gp120, whereas on functional envelope spikes this is counteracted by oligomerization.

[0168] The opposite effect between binding to monomeric gp120 and neutralization was observed with mutants N276A and S365A than for the previous five mutants; i.e., although binding to monomeric gp120 was increased, neutralization efficiency was lost (neutralization indices were 0.44 and 0.04, respectively). Residue N276 is an *N*-linked glycosylation site that is part of the D-loop on gp120 (61), which is believed to provide important residues for b12 binding (152, 153). Removal of the glycan may facilitate the interaction of b12 with monomeric gp120, whereas on the native trimer, the absence of the glycan may adversely affect the conformation of the D-loop (61) and lead to the observed decrease in neutralization efficiency. Residue S365 is part of a ridge formed by residues 364-368 that make direct contact with CD4 (61) and, based on the b12-gp120 docking model, may fit into a cleft formed by the heavy chain CDR2 and CDR3 of b12 (90). The S365A mutation, like the N276A mutant, may cause a conformational change on monomeric gp120 that favors b12 binding to monomer, but that has a negative effect on b12 binding to functional oligomeric gp120.

[0169] A feature of b12 neutralization escape mutants selected *in vivo* is a mutation at position 369 (Pro) to Gln or Leu (74, 104). When mutated to alanine (P369A), no change in binding affinity for monomeric gp120 and no significant change in neutralization sensitivity

was observed (Table 2, below), indicating that this residue does not normally form part of the b12 epitope. Rather, it is more likely that mutating this amino acid to a larger residue caused the steric impairment of the interaction between b12 and gp120, either directly or by altering peptide backbone conformation.

[0170] Based on the mutagenesis results, four recombinant gp120 molecules containing multiple alanine mutations between amino acid residues G473-R476 were generated and tested on a panel of five non-neutralizing anti-CD4bs mAbs, b12, and CD4 (Table 4, below). The quadruple mutant GDMR increased b12 binding, but completely abolish binding by three (b3, b6, F105) of the five non-neutralizing anti-CD4bs mAbs tested. Of the two remaining mAbs, the binding affinity of one mAb, 15e, was reduced for two of the four mutants, whereas with the other mAb, F91, a slight decrease in binding affinity was observed. These results demonstrate that gp120 can be engineered so as to make it less prone to recognition by non-neutralizing antibodies.

EXAMPLE 2

HYPERGLYCOSYLATED MUTANT gp120 POLYPEPTIDES

[0171] This example demonstrates that mutation of gp120 polypeptide sequences to generate glycosylation sites, and glycosylation of such mutant polypeptides, reduces or inhibits non-neutralizing antibody binding.

[0172] The ability to induce broadly neutralizing antibodies should be a key component of any forthcoming vaccine against human immunodeficiency virus type 1. One potential vaccine candidate, monomeric gp120, has generally failed to elicit such antibodies. As disclosed in Example 1, above, a better HIV immunogen was prepared by engineering gp120 to preferentially bind known broadly neutralizing antibodies. For example, four alanine substitutions on the perimeter of the Phe43 cavity of gp120 reduced binding of weakly neutralizing CD4-binding site antibodies, while slightly enhancing binding of the potent, broadly neutralizing antibody b12.

[0173] The present example extends the results described in Example 1 by introducing *N*-glycosylation motifs at select positions into the hypervariable loops and the gp120 core,

and examining the mutant glycosylated gp120 polypeptides for the ability to further reduce or abrogate the binding of a wider range of non-neutralizing antibodies. As disclosed herein, a hyperglycosylated mutant gp120 polypeptide containing seven extra glycosylation sequons and four alanine substitutions as described in Example 1 did not bind an extensive panel of non-neutralizing and weakly neutralizing antibodies, including a polyclonal immunoglobulin preparation (HIVIG) of low neutralizing potency. Binding of b12, at lowered affinity, and of four antibodies to the C1 and C5 regions was maintained. Removal of N-terminal and C-terminal residues in the C1 and C5 regions, respectively, reduced or abolished binding of the four antibodies, but also adversely affected b12 binding. Thus, the hyperglycosylated mutant gp120 polypeptides and analogues thereof provide previously undescribed antigens that can provide a new approach to eliciting antibodies with b12-like neutralizing properties.

MATERIALS AND METHODS

Plasmids and mutagenesis

[0174] The generation of plasmid pCMV-Tag4A-tpa_{JR-FL}gp120_{wt} has been described (94). This plasmid, which is derived from plasmid pCMV-Tag4A (Stratagene), contains a tissue plasminogen activator leader sequence immediately upstream of the *env* gene to ensure secretion of gp120 envelope glycoprotein into the culture supernatant. The *env* gene of HIV-1 JR-FL was obtained by PCR amplification using as template a plasmid (pSyngp140_{JR-FL}) encoding the codon-optimized gp140 gene (2, 45) of this HIV isolate. Site-directed mutagenesis to substitute wild-type residues for alanine and to incorporate *N*-glycosylation sequence motifs was performed using the QUIKCHANGE mutagenesis kit (Stratagene).

[0175] The generation of gp120 containing deletions of residues in the N and C termini (52 and 19 residues, respectively) was performed by PCR. The primers fICORE-5 (5'-GGAGGTCAACAGCACCG**CGCG**GAGGTGGTGCTGGAGAATGTGAC-3'; SEQ ID NO:13), which contains a *Bss*H II restriction site (bold), and fICORE-3 (5'-GGAGGTCAACAGCACCC**CTCGAG**TTAATTAATTAAC**TCAATCTTCACCACTT**GTA-3'; SEQ ID NO:14), which contains an *Xho* I site (bold) were used. Plasmids

encoding wild-type or mutant gp120 were used as templates. The PCR products were cloned into pCMV-Tag4A-tpa using restriction enzymes *Bss*H II and *Xho* I according to standard protocols. DNA sequencing prior to use verified that the plasmids and mutations generated in this study were correct.

Antibodies

[0176] A total of 22 mAbs and one polyclonal immunoglobulin preparation were used. MAbs b3, b6, b12, X5 and loop 2 were as described (6, 7, 20, 22, 83, 110). The remaining antibodies were obtained from their respective source or from the NIH AIDS Research and Reference Reagent Program (NIH ARRRP). Primary citations for these mAbs are as follows: C11, 212A, 19b, 17b, 48d, F91, 15e, A32 (49, 76-78, 81, 82, 111, 112, 123, 133, 134, 144); 2G12 (16, 137); G3-4, G3-136 (36, 48, 132); 447-52D (17, 41, 42); F105 (105, 106, 135); M90 (33); 133/290 and 133/237 (71, 82, 87); 522-149 (80). The epitopes recognized by the antibodies are summarized in the HIV sequence database (see hypertext transfer protocol ([http](http://resdb.lanl.gov/ABDB/antibody_id.htm)), at URL "resdb.lanl.gov/ABDB/antibody_id.htm", which is incorporated herein by reference). Human IgG purified from pooled plasma obtained from healthy asymptomatic seropositive individuals (HIVIG) was obtained from the NIH ARRRP. This manufactured product (NABI, Boca Raton, Florida) is a 50 mg/ml solution that contains 98% monomeric IgG (28). An affinity-purified polyclonal antibody preparation against the C5 region of gp120, APTKAKRRVVQREKR (SEQ ID NO:15), was purchased from Cliniqua (Fallbrook, CA).

Transient expression of recombinant HIV glycoproteins

[0177] Monomeric wild-type gp120 and mutant glycoproteins were obtained by transiently transfecting 293T cells (94). Culture supernatants containing recombinant glycoproteins were stored at -20°C.

MAb biotinylation

[0178] MAb 2G12 was conjugated to biotin using EZ-Link biotinylation reagents (Pierce) according to the manufacturer's instructions. Biotin-labeled mAb was tested in ELISA to assure that the conjugation did not adversely affect binding to gp120.

Enzyme-linked immunosorbent assays (ELISA)

[0179] Assays were performed essentially as described (94). Glycoproteins were captured onto ELISA plate wells using the anti-C5 polyclonal antibody preparation, unless otherwise indicated. Antibodies against CD4-induced epitopes were tested in the absence of soluble CD4. MAb 2G12, or in some cases HIVIG, was used to ensure that similar amounts of envelope proteins were captured in each experiment. In general, plates were developed with p-nitrophenyl phosphate (Sigma) and absorbance measured at 405 nm. When peroxidase-conjugated secondary antibody (Pierce) was used, plates were developed with 3, 3', 5, 5' tetramethyl benzidine/hydrogen peroxide substrate (TMB, Pierce) and absorbance measured at 450 nm. In this case, the color reaction was stopped with sulfuric acid (2 M) prior to measurement. For detection of biotinylated 2G12, peroxidase-conjugated streptavidin (Jackson ImmunoResearch) was used in combination with the TMB system. ELISA assays were performed in duplicate and repeated on at least two different occasions.

SDS-PAGE and Western blot analysis

[0180] SDS-PAGE and western blot analyses were performed essentially as described (93, 140). For electrophoresis, supernatants from 293T cells transiently expressing wild-type or mutant glycoproteins (typically one 6-well plate for each glycoprotein) were pooled and concentrated ~10 times using YM-50 concentrators (Amicon). The supernatants were diluted 1:2 with concentrated sample buffer (125 mM Tris/HCl, 4% SDS, 20% glycerol, 0.02% bromphenol blue; pH 6.8) and vortexed briefly. Of this mixture, 10 μ l were loaded

onto a 5% separating gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane by tank blotting (BioRad). Blots were incubated overnight with primary antibody (1 or 5 μ g/ml, as indicated), then with alkaline phosphatase-conjugated secondary antibody (Pierce) and developed using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (BCIP, NBT) as substrates (Sigma).

RESULTS

Effect of multiple alanine substitutions on the perimeter of the Phe43 cavity of gp120 on mAb binding

[0181] As disclosed in Example 1, the effects of multiple alanine substitutions on the perimeter of the Phe43 cavity (positions 473-476) of gp120_{JR-FL} on the binding of a panel of seven CD4bs antibodies was examined. For one mutant, GDMR, in which four gp120 amino acid residues were substituted by alanine, binding of mAbs b3, b6, CD4-IgG2 and F105 was essentially abolished, whereas the apparent binding affinity of mAbs F91 and 15e was reduced relative to wild type. In contrast, the binding affinity of mAb b12 was unaffected or slightly enhanced.

[0182] In the present example, a larger panel of antibodies was tested with the GDMR mutant. A total of 14 anti-gp120 antibodies were selected, including the seven antibodies described above. One of the antibodies, mAb A32, binds to a discontinuous epitope involving the C1-C4 domains on gp120 and competes with CD4bs antibodies for binding (80, 144). The affinity of this antibody is increased in the presence of soluble CD4, thus qualifying it nominally as a "CD4-induced" antibody. However, mAb A32 does not compete with other CD4-induced mAbs, e.g. 17b and 48d, for binding to gp120 (144). The other antibodies bind to continuous and discontinuous epitopes in the C1 and C5 domains of gp120 (mAb C11), the V2 loop (mAbs G3-4 and G3-136), and the V3 loop (mAbs loop 2, 19b and 447-52D). To determine apparent binding constants, cell culture supernatants containing recombinant glycoproteins were captured onto ELISA plate wells using a polyclonal antibody preparation to the C5 region of gp120. The specificity of this polyclonal antibody is similar to that of mAb D7324, and it does not compete for binding with any of the antibodies tested here (80). Captured glycoproteins were then probed with

varying concentrations of antibody to generate binding curves. Apparent affinities were determined from the antibody concentration at half-maximal binding, and related to that determined for wild-type gp120.

[0183] Most antibodies bound with high affinity to wild-type gp120_{JR-FL} (Figures 7A and 7B). However, mAbs G3-4 and G3-136, which recognize similar conformational epitopes in the V2 loop, and mAb C11 bound with only moderate affinity to monomeric gp120_{JR-FL}. The moderate binding affinity of mAbs G3-4 and G3-136 to gp120_{JR-FL}, in contrast to binding to gp120_{IIIB}, was likely due to sequence differences between the two gp120s. The sequence -STSIRGKVKEYAFFYKLDI- (SEQ ID NO:16), which is believed to be involved in antibody binding to gp120_{IIIB} (101, 150), is replaced by the sequence -**TTSIRDEVQKEYALFYKLDV**- (SEQ ID NO:17) in gp120_{JR-FL} (residues in JR-FL that differ from those in IIIB are in bold). The indicated residues can cause slight changes in the epitope recognized by these two antibodies, thus diminishing antibody binding. The same may hold true for mAb C11, although the epitope of mAb C11 has not been clearly defined.

[0184] As expected, mAbs b3, b6 and F105 did not bind to gp120 of mutant GDMR, whereas the binding of mAbs F91 and 15e was reduced relative to wild type (55% and 45%, respectively; Figure 7C). The binding of mAb A32 was slightly reduced (70% relative to wild-type gp120; Figure 7D). In contrast, binding of the other antibodies, including b12, was largely unaffected by the alanine substitutions (Figure 7C and 7D). Based on these results, it was apparent that, although introduction of the four alanine mutations was sufficient to abrogate or reduce binding to gp120 of non-neutralizing or weakly neutralizing mAbs against epitopes close to or overlapping the CD4bs, additional mutations can further eliminate the reactivity of undesired antibodies against other gp120 epitopes.

Effect of the introduction of an *N*-linked glycan in the V3 loop on reactivity with V3 loop antibodies

[0185] First, it was determined whether introduction of an *N*-glycan in the V3 loop could inhibit reactivity by V3 loop mAbs. For this purpose, a mutant, termed P313N, was generated. In the P313N mutant, which also included an R315T substitution

(P313N/R315T), Arg and Thr were substituted for Pro and Arg at positions 313 and 315, respectively, in the "tip" of the V3 loop (-Gly-Pro-Gly-Arg-Ala-Phe-; SEQ ID NO:17) segment. This *N*-glycosylation sequon (NXT) permits the addition of a potential *N*-linked glycan at position 313. SDS-PAGE and western blotting were used to investigate the presence of the extra glycan (Figure 8A); a slight increase in average molecular weight was observed for the mutant glycoprotein in comparison to wild-type gp120, suggesting the presence of an additional glycan in the mutant.

[0186] The effect of the mutation on antibody reactivity was determined for three V3 loop mAbs, namely loop 2, 19b and 447-52D. These three antibodies pooled together were unable to react with glycoprotein of mutant P313N as determined by western blot (Figure 8B). ELISA, using individual mAbs, confirmed these results (Figure 8C and 8D). In contrast, the apparent affinity of mAb b12, relative to wild type, was unaffected by the introduced mutation. The glycoprotein of mutant P313N was stained much more strongly by b12 in comparison to wild-type gp120 (Figure 8A), perhaps due to a difference in the amount of glycoprotein present in the respective preparations, which were not standardized prior to SDS-PAGE. To determine whether the inability of the three V3 loop antibodies to bind mutant P313N was due solely to incorporation of an *N*-linked glycan, the Asn at position 313 was replaced by Gln (the Arg-to-Thr substitution at position 315 was left unchanged); the three antibodies were also unable to react with this mutant, indicating that, even though the V3 loop mAbs may be blocked from binding to their respective epitopes by the introduced glycan, reactivity also is abrogated due to substitution of the proline residue alone or in combination with the arginine residue. The mutations most likely disrupt the ϵ -turn at the apex of the V3 loop comprising the -Gly-Pro-Gly-Arg- segment (40, 129).

Effect of introducing *N*-linked glycans in gp120 on reactivity with anti-gp120 mAbs

[0187] Next, other epitopes that could be recognized by non-neutralizing antibodies were blocked using the same glycosylation strategy. First, *N*-glycosylation sequons were introduced individually at various positions in gp120, primarily on the non-neutralizing face (143) and in the V1 and V2 loops, to determine the effect on b12 binding. The NXT glycosylation sequon (X is any amino acid except proline) was used because the asparagine

in this motif is 2-fold more likely to be glycosylated than the asparagine in the NXS motif (124). In some cases two substitutions, at the 'N' and 'T' positions, were required to introduce the glycosylation motif, whereas in other instances only one of the two positions needed to be substituted by the appropriate residue. The mutations incorporated were as follows: Q114N/L116T and Q246N (masking of the non-neutralizing face and the putative gp41-gp120 interface), S143T and E150N/G152T (masking of the V1 loop), K171N/Y173T (masking of the V2 loop), S365T (masking of potential epitopes on the outer perimeter of the CD4bs) and R440N/Q442T (masking of potential epitopes close to the base of the V3 loop, adjacent to the silent face). The S143T and S365T mutations were introduced to enhance the likelihood of glycan attachment at residues N141 and N363, respectively.

[0188] Binding of mAb b12 was not significantly affected by mutations Q114N/L116T and S143T, whereas binding was somewhat decreased by the E150N/G152T mutation (Figure 9). Binding was more strongly affected by the mutation in the V2 loop (K171N/Y173T) and by the Q246N mutation. With the two remaining mutants, S365T and R440N, no reactivity with b12 was observed. Expression of the mutant with the R440N/Q442T substitution was very poor, suggesting that introduction of a glycan at position 440 interferes with the proper folding or intracellular processing of gp120.

[0189] Glycosylation motifs that did not affect b12 binding, or affected it only slightly, i.e., mutations Q114N/L116T, S143T and E150N/G152T, were then combined. The P313N and R315T substitutions (mutant P313N), respectively, and the alanine substitutions at positions 473-476 (mutant GDMR; see Example 1) were also incorporated to generate a hyperglycosylated mutant (Figure 10). In addition, the K171N/Y173T mutation was inserted, despite its negative effect on b12 binding, because this was the only mutation in the V2 loop that could potentially hinder the binding of V2 loop antibodies. Additional *N*-glycosylation sites were also introduced at positions 92 and 423, without prior testing with b12. Residue H92 lies on the non-neutralizing face of gp120 and residue I423 lies in the coreceptor-binding site.

[0190] Western blots were performed to confirm that the glycoprotein of this mutant, termed mCHO*-GDMR, was hyperglycosylated. The molecular weight of the modified glycoprotein was ~137 kDa (Figure 11), suggesting that most, if not all, extra glycosylation sites are occupied in this mutant. The largest oligosaccharide that can be incorporated at each site is a fully sialylated, fucosylated and galactosylated tetraantennary complex glycan (~3.5 kDa), which would increase the average molecular weight of wild-type gp120 by ~25 kDa. However, this complex glycan type is observed in less than 1% of the total glycans on gp120_{JR-FL} (119). Rather, recombinant gp120_{JR-FL} expressed in mammalian cells appears to contain predominantly galactosylated, fucosylated biantennary complex glycans (sialylated and non-sialylated) and high oligomannose glycans (Man-8) (119). Such glycans would add an average of ~2 kDa per glycosylation site, thus increasing the molecular weight of wild-type gp120 to ~134 kDa, which approximates the average mass determined from the SDS gel for the mutant mCHO*-GDMR. However, the possibility that some sites are not glycosylated cannot be excluded from this determination; mass spectrometric analyses of the mutagenized protein (151) can address this issue in detail.

[0191] To investigate the antigenic properties of this glycoprotein mutant, binding affinities were determined for b12 and for a large panel of non-neutralizing anti-gp120 mAbs. Most mAbs bound wild-type gp120_{JR-FL} with high affinity (Figure 12A to 12D). Saturation levels were not obtained with certain mAbs against the C1 domain (133/237, 133/290 and 522-149) and, as already observed, by mAbs C11, G3-4 and G3-136. MAb 133/237 and 133/290 bind better to denatured gp120 than to native glycoprotein (79) and here display moderate affinity for gp120 in ELISA. Whether this is also the case for mAb 522-149 is not known. It is also possible the moderate binding affinity stems from sequence differences between the homologous antigen and gp120_{JR-FL}. Antibodies 17b and 48d also bound with lower affinity to wild-type gp120, due to the absence of sCD4 in these experiments. When tested with mutant mCHO*-GDMR, binding of virtually all mAbs was abolished, whereas binding of mAb b12 was reduced in comparison to wild-type gp120. Unfortunately, but not unexpectedly, the binding of only two of the five C1 antibodies was inhibited. In this study, no glycan attachment motifs were introduced in the C1 region

because glycosylation of the N-terminus is reported to be rare (39). Binding of the three other C1 antibodies to mutant mCHO*-GDMR was nearly twofold higher than to wild-type gp120 (Figure 12E). Removal of N-terminal residues in the C1 region reduced or abolished the ability of these antibodies to bind gp120, and deletion of residues in the C-terminus of the C5 region reduced the binding of the anti-C5 polyclonal antibody preparation used to capture gp120 (Figure 13). Surprisingly, b12 binding was also severely diminished (Figure 13), indicating that the combined removal of these N-terminal and C-terminal residues dramatically influences the conformation of the b12 epitope in mutant mCHO*-GDMR.

Epitope masking by introduction of *N*-glycans abolishes reactivity with a polyclonal antibody preparation

[0192] To test the degree to which this approach can mask non-neutralizing epitopes on gp120, the effects of the introduced glycans on binding of a preparation of polyclonal antibodies was examined. This preparation, HIVIG, which is derived from pooled plasma of asymptomatic individuals that was selected based on high antibody titers to the HIV structural protein p24, neutralizes primary HIV-1 isolates poorly (28). When tested with mutant GDMR, a 2-fold reduction in relative binding affinity was observed, in comparison to wild-type gp120_{JR-FL} (Figure 14). However, binding was much more reduced to the mutant with an extra glycosylation site in the V3 loop, thus suggesting the presence of a large percentage of V3 loop antibodies in this preparation. No binding of HIVIG to mutant mCHO*-GDMR was observed, indicating that a number of epitopes on gp120 that have the potential to induce non-neutralizing antibodies successfully were masked.

Influence of the *N*-glycosylation motif in the V2 loop on b12 binding

[0193] To examine whether the lowered affinity of mAb b12 for mutant mCHO*-GDMR was caused by the K171N/Y173T mutation in the V2 loop, the mutation was reverted to wild-type sequence. Antibody b12 binding affinity for this mutant, termed K171Nx, was similar to that for mutant mCHO*-GDMR. Therefore, the lower affinity of b12 for mutant mCHO*-GDMR in comparison to wild-type glycoprotein likely stems from the introduction of a particular combination of glycans in mutant mCHO*-GDMR, rather

than from steric hindrance by a specific glycan. Interestingly, the V2 loop antibody G3-4 did not react with mutant K171Nx, indicating that the extra V2 loop-glycan may not be required for masking antigenic determinants in this loop. Rather, the result with mAb G3-4 suggests that neighboring glycans that were inserted are sufficient to block the binding of V2 loop antibodies.

Influence of alanine substitutions at positions 473-476 in mutant mCHO*-GDMR on binding of mAbs to epitopes overlapping the CD4bs

[0194] Although epitopes of various non-neutralizing and weakly neutralizing antibodies were successfully masked, it was unclear whether blockage of CD4bs antibodies and mAb A32 could be accomplished by the glycans alone, i.e., without the need for the introduced alanine substitutions at positions 473-476. Therefore, another variant of mutant mCHO*-GDMR was generated. In this variant, termed mCHO*, the alanine substitutions at positions 473-476 were reverted to wild type. As shown in Figure 15, binding of mAbs 15e, F91, F105 and A32 was significantly reduced or completely abolished, indicating that the added glycans, alone, were sufficient to block binding of many non-neutralizing or weakly neutralizing CD4bs antibodies; the weakly neutralizing antibodies b3 and b6 were able to bind the mCHO* mutant glycoprotein, albeit with lower affinity than mAb b12. Thus, the extra glycans, while not completely blocking access to the CD4bs, appear to have confined the space available to antibodies for interaction with this site.

[0195] A major concern for HIV-1 vaccine design is that at present no immunogen, or combination of immunogens, is capable of eliciting the levels of broadly neutralizing antibodies that are likely needed to contribute to significant protection against infection. Nevertheless, the anti-gp120 and anti-gp41 mAbs that have been isolated from natural infection and from immunization studies serve as valuable tools to screen prospective antigenic formulations for their suitability as candidate immunogens (19, 145). However, antigens that preferentially bind neutralizing antibodies, but not non-neutralizing ones, would be most desirable. As disclosed in Example 1, binding of the broadly and potently neutralizing human antibody b12 was unaffected or slightly enhanced by the introduction of four alanine mutations on the perimeter of the Phe43 cavity on gp120 (mutant GDMR),

whereas binding of CD4 and five weakly neutralizing CD4bs antibodies was abolished or reduced (see, also, (94)).

[0196] As disclosed herein, the GDMR gp120 mutant was further examined. As a first step, mutant GDMR was tested in ELISA with a selection of mAbs against various linear and discontinuous epitopes on gp120. Only non-neutralizing or weakly neutralizing CD4bs mAbs were significantly affected by the alanine substitutions (Figure 7). It is unclear whether this result correlates with how these antibodies interact with gp120 and their lack of neutralizing potency. However, considering that antibodies often form salt-bridges or hydrogen bonds with available polar groups on the antigen, most probably because this compensates for the loss in entropy upon antigen interaction (10, 14, 15, 126, 138, 139), it is noteworthy that mAbs b3, b6 and F105 are particularly sensitive to substitution of residues at positions 474 (Asp) and 476 (Arg) by alanine (94). The results obtained using the GDMR mutant suggest that these aspartate and arginine residues are ideal contact residues for many non-neutralizing antibodies. These residues may be more accessible on monomeric gp120 than on the trimeric envelope spikes of primary HIV isolates, thus explaining why these antibodies are unable to neutralize virions potently.

[0197] The observation that non-CD4bs antibodies were not inhibited from binding to mutant GDMR prompted the pursuit of further means for blocking epitopes recognized by non-neutralizing antibodies. The concept of diverting B-cell immune responses away from undesired epitopes has been discussed (32). A recent study (24) showed that, by introducing a single amino acid mutation in the ϵ -chain of human chorionic gonadotrophin, cross-reactive antibodies to lutenizing hormone, which are normally elicited upon immunization with wild-type human chorionic gonadotrophin ϵ -chain, could be eliminated. As such, the immune response was refocused to epitopes that are normally only weakly immunogenic.

[0198] In the case of HIV, such a strategy is likely to be tedious and time-consuming, particularly considering the vast genetic diversity that is manifested among HIV isolates (8). Therefore, a decision was made to insert *N*-glycosylation sequons into the gp120 sequence

with the aim of selectively incorporating additional *N*-glycans onto the glycoprotein to mask undesired epitopes. Masking epitopes in the (hyper)variable loops of HIV was of particular importance, since antibodies to such sites are often induced upon immunization with gp120 or during natural infection (25, 34, 43, 64, 67, 70, 75, 122, 127, 141). During infection, these antibodies are detected relatively early, suggesting the presence of immunodominant epitopes within the loops (34, 70, 86, 122). The masking of epitopes by glycans is not, in itself, novel and, in fact, is a strategy employed by HIV to avoid facile recognition by the host immune system (5, 31, 92, 107, 108, 120, 143). For example, a large portion of gp120 is covered by *N*-glycans, thus rendering those regions of the antigen immunologically 'silent' (143). Furthermore, changes in the number and placement of *N*-linked glycans in gp120 can modulate the exposure of antigenic determinants. For example, elimination of an *N*-glycan in the V3 loop by site-directed mutagenesis increases viral sensitivity to neutralizing antibodies (5). Also, when virus lacking an *N*-linked glycan in the V3 loop is grown in the presence of a V3 loop antibody, it rapidly reverts to a variant in which a glycan is reincorporated at that position (120). However, in terms of vaccine design, the approach of epitope masking by the incorporation of additional *N*-linked glycans is not widespread and, in fact, has been applied in only one previous study, wherein incorporation of an *N*-glycosylation site in the V3 loop resulted in a shift in the immune response towards epitopes in the V1 loop (37).

[0199] In the present study, *N*-linked glycans were introduced based primarily, but not solely, on the following criteria: (i) the side chain of the residue to be mutated should be solvent-exposed, (ii) the introduced glycan should block a region on gp120 that might elicit non-neutralizing antibodies, (iii) the site selected for introduction of the *N*-glycan should contain residues that might tolerate mutation, yet still retain a similar conformation, and (iv) the potential glycosylation site should not be hindered by neighboring sequence elements. For incorporation of the *N*-glycans, an NXT glycosylation sequence was selected rather than an NXS motif because the asparagine in the NXT sequon is more likely to be glycosylated (39, 52, 73, 124). Furthermore, in an NXT glycosylation motif, more residues

are tolerated at position X in terms of glycosylation efficiency compared to an NXS sequence (52).

[0200] First, a determination was made whether introduction of an *N*-glycan in the V3 loop could abolish binding of V3 loop antibodies. Considering that a glycan at the apex of V3 may potentially mask a substantial portion of the loop on both sides, a glycosylation site was incorporated at position 313 (Pro). Indeed, none of the three V3 loop mAbs tested were able to bind to this mutant. However, antibody binding to a second mutant, in which the Asn in the glycosylation sequon was replaced by Gln, was also abrogated. These results indicate that incorporation of the extra glycosylation sequon serves two functions. First, incorporation of the glycosylation motif allows potential antibody epitopes to be masked due to the presence of the glycan. Whether the glycan indeed masks the entire loop is uncertain, since there are currently no antibodies available that are reactive with residues down- or upstream from the introduced glycosylation site in the V3 loop of JR-FL. The second effect of replacing the proline and arginine residues is that this most likely eliminates or modifies the ϵ -turn and ϵ -type hairpin, which are characteristic of the apex of the V3 loop and confer immunodominance to the loop (40, 109). Lowering this predominant characteristic of the V3 loop may further increase the potential to obtain antibodies against epitopes on gp120 that are normally only weakly immunogenic.

[0201] Next, the glycosylation strategy was extended to other epitopes that could elicit non-neutralizing antibodies. Seven sites were selected for the incorporation of *N*-glycosylation motifs (Figure 9). The mutations were generated in individual mutants first and tested with b12, to ensure that none severely compromised gp120 folding as detected by antibody binding. Of the seven mutations, three were eliminated from subsequent studies because they abolished b12 binding. The added glycosylation site in the V2 loop also reduced b12 binding, but was still selected for further studies since no other glycosylation motifs had been incorporated in the V2 loop for epitope masking. This mutation and the others that did not significantly affect b12 binding were combined together with the glycosylation sequon in the V3 loop and the alanine substitutions in the Phe43 cavity to generate a hyperglycosylated mutant glycoprotein. Two additional glycosylation motifs

were introduced at positions 92 and 423, to further mask the non-neutralizing face on gp120 and the coreceptor-binding site, respectively. This hyperglycosylated gp120 mutant, mCHO*-GDMR, blocked the binding of virtually all non-neutralizing and weakly neutralizing antibodies examined, including a polyclonal antibody preparation (HIVIG) of low neutralizing potency. Importantly, b12 binding was maintained, albeit at lowered affinity. However, this reduced binding was not caused by the *N*-glycan that was incorporated in the V2 loop, since reversion to the wild-type sequence did not alter b12 affinity. Binding to mutant mCHO*-GDMR was also still observed with three mAbs against the C1 region and a polyclonal antibody against the C5 region. Although the binding of these antibodies could be reduced or abolished by removal of residues in C1 and C5, respectively, b12 binding was also severely reduced (Figure 13). Thus, in the mutant mCHO*-GDMR, b12 binding can not be maintained at the expense of the remaining C1-reactive and C5-reactive antibodies by truncating the N and C termini because of the apparent negative effect on the conformation of the b12 epitope.

[0202] In summary, a series of mutant gp120 polypeptides have been generated that diminish or abolish the binding of non-neutralizing or weakly neutralizing antibodies to varying degrees, but retain, at least to some extent, b12 binding. The mutant gp120 polypeptides have the potential to induce broadly neutralizing antibodies, particularly those targeted to the CD4bs. Although it may be considered that, because of the extra glycans, the mutant gp120 is conformationally different from the gp120 conformation on the viral surface, the ability of b12 to bind to mutant mCHO*-GDMR indicates that it is unlikely that the extra oligosaccharide moieties significantly alter the overall gp120 core structure, including the CD4bs. Since the majority of broadly neutralizing antibodies appear to bind equally well to monomeric and oligomeric gp120 (35, 99, 116), antibodies that recognize the CD4bs on gp120 in a b12-like manner should also be reactive with oligomeric gp120. In fact, the introduction of the extra *N*-glycans may have created spatial constraints around the CD4 binding domain that antibodies are likely to encounter with native, virion-associated oligomeric gp120.

[0203] Besides epitope masking, the extra glycans also can serve an additional function. Recent studies suggest that monomeric gp120 contains unusually high intrinsic entropy, which might undermine efforts to obtain broadly neutralizing antibodies (84). Considering that asparagine-linked glycosylation influences protein structure and folding (51, 52, 89, 142) and that the addition of carbohydrate moieties may minimize the conformational flexibility of proteins (89, 142), the extra glycans incorporated into the gp120 mutants can reduce the overall flexibility of monomeric gp120 and, therefore, further promote the induction of antibodies with neutralizing properties superior to those obtained with unmodified gp120.

[0204] Accordingly, a panel of mutant gp120 polypeptides is provided that, based on their antigenic properties, can be useful as immunogens for stimulating primarily a neutralizing antibody response.

TABLE 1. Alanine and variable loop-deleted mutants generated in this study and their binding to mAbs and CD4.

Domain ^a	Mutant ^b		Apparent affinity relative to wild-type gp120 _{JR-CSF} (%) ^c for:				
			b3	b6	b12	CD4 ^d	2G12
C1	E87A	○	44	24	7	114	ND ^e
	M95A	○	6	100	75	59	ND
	K97A	○	94	111	134	50	ND
	E102 ^f A	○	52	167	63	47	ND
	W112 ^f A	○	80	17	2	67	ND
	D113 ^f A	○	154	8	1	9	169
C1 (V1/V2-stem)	K121 ^g A	○	154	19	4	3	ND
	L122 ^g A	○	60	22	27	62	ND
	T123 ^g A	●	63	74	233	42	ND
	L125 ^{g,h} A	○	18	360	383	23	ND
	V127 ^g A	○	5	46	62	26	ND
V2	I165A		30	19	20	57	ND
	R166A	○	40	23	52	44	ND
	K171A	○	123	100	118	26	ND
	E172A		9	130	143	93	ND
	F176A	○	3	4	2	19	400
	D180A	○	31	7	1	31	108
	I184A	○	34	7	0.6	27	114
	D185A		48	63	6	62	ND
	T190A		300	10	21	187	ND
C2 (V1/V2-stem)	N197 ^g A	○	8	83	25	3	318
	T198 ^f A	○	80	17	0.1	42	ND
	S199 ^g A	○	10	143	123	4	ND
	T202A	○	63	500	220	62	ND
C2	K207 ^g A	○	10	3	2	5	100
	F210A	○	229	29	77	37	ND
	I213 ^g A	○	40	15	20	31	114
	R252A		600	12	17	47	ND
	S256 ^g A	●	60	250	13	47	108
	T257 ^g A	●	20	42	28	21	133
	N262 ^g A	○	71	10	6	5	ND

	R273A	○	67	5	0.9	40	ND
	N276A	○	5	260	225	27	169
	D279 ^{f,h} A		12	93	27	33	ND
	N280 ^h A	○	114	93	75	44	ND
	K282 ^f A	○	114	25	32	78	ND
	T283 ^h A	○	80	250	55	53	ND
C3	Q337A		185	500	83	122	ND
	K343A		80	147	8	187	ND
	R350A		11	9	6	43	133
	S365 ^h A	○	40	590	534	23	1800
	G366 ^{f,h} A	○	89	36	9	1	ND
	G367 ^h A	●	120	7	1	2	ND
	D368 ^{f,h} A	●	1	130	15	1	ND
	P369A		160	167	129	117	ND
	E370 ^h A	○	1	0.8	0.2	4	ND
	I371 ^{f,h} A	○	40	31	26	7	ND
	V372A		100	55	4	52	ND
	M373A		91	12	13	80	ND
	Y384 ^g A	○	31	13	2	40	ND
	N386 ^f A	○	16	20	11	26	21'
	T388 ^f A		120	25	8	19	ND
V4	W395A	○	8	14	18	23	25'
C4	K421 ^g A	○	63	12	7	44	ND
	N425 ^h A	○	40	138	80	11	ND
	M426 ^h A	○	20	40	0.2	0.2	133
	W427 ^{g,h} A	○	300	57	21	9	ND
	V430 ^h A	○	145	500	580	2	ND
	K432A		111	20	7.5	50	ND
	I439 ^f A	○	15	6	2	10	133
	T450 ^g A	●	9	8	7	16	108'
	T455 ^{f,h} A	○	1	464	500	1	ND
	R456 ^h A	○	87	13	6	53	ND
	D457 ^{g,h} A	○	120	37	5	2	ND
	G458 ^h A	○	53	333	300	2	ND
	G459 ^h A	○	171	80	54	37	ND
	N461A		87	17	4	160	ND

V5	E462A		20	52	21	32	ND
	S463A		300	100	50	53	ND
	I467A		40	300	63	3	ND
	R469 ^{f,h} A	○	154	5	1.4	8	ND
	P470 ^f A	○	44	36	1.3	13	100
	G471A	○	74	250	37	89	ND
C5	G472 ^h A	○	20	25	22	2	100
	G473 ^{f,h} A	○	9	175	998	2	ND
	D474 ^{f,h} A	○	0.1	0.3	91	22	ND
	M475 ^f A	○	20	27	130	64	ND
	R476 ^f A	○	0.4	0.2	25	40	133
	D477 ^f A	○	86	31	2	187	ND
	W479 ^g A	●	1	6	1.3	19	40
V1-V3	R480 ^f A	○	57	63	37	73	ND
	ΔV1		23	33	35	44	133
	ΔV1/2		6	18	30	12	114
	ΔV3		75	29	30	41	ND

^agp120 domain. C, constant domain; V, variable loop.

^bAmino acid numbering is relative to HIV-1_{HXB2}, where 1. is the initial methionine (57). Δ denotes amino acid deletions: ΔV1 (amino acids 134-154); ΔV1/V2 (amino acids 134-154 and 160-193); ΔV3 (amino acids 303-324). White circles (○) indicate that an amino acid is identical among 51-98% of all HIV-1 isolates. Black circles (●) indicate that an amino acid is identical among 99-100% of all HIV-1 isolates (amino acid identity determined from sequence alignment of HIV-1 isolates listed in the HIV sequence database at <http://hiv-web.lanl.gov/content/hiv-db/mainpage.html>).

^cApparent affinities were calculated as the antibody concentration at half-maximal binding. Apparent affinities relative to those for wild-type gp120 were calculated with the formula: (apparent affinity for the wild type/apparent affinity for the mutant) x 100. The color scheme is the same as in Fig. 3; substitutions which resulted in apparent affinity <50% relative to wild type are colored blue, those which resulted apparent affinity between 50-200% relative to wild type are colored grey, and those that resulted in apparent affinity >200% relative to wild type are colored yellow.

^dCD4-IgG2 was used as a surrogate for CD4 in this study.

^eND, not determined.

^fAmino acid residues conserved among all HIV-1 isolates. Amino acid conservation is defined as in reference (61): single amino acid changes are allowed, as are larger substitutions, as long as the character of the side chain is maintained (e.g., Lys to Arg or Phe to Leu).

^gAmino acid residues conserved among all primate immunodeficiency viruses.

^hCD4 contact residues [as determined from the crystal structure of the gp120-CD4-17b complex by Kwong et al. (61)].

ⁱData derived from reference (119)

Table 2. Neutralization of wildtype and mutant pseudovirions of HIV-1_{JR-CSF} by mAb b12.

Pseudovirus	IC ₉₀ ^a	Apparent affinity relative to wildtype gp120 _{JR-CSF} (%)	Neutralization index ^b
wildtype	14	100	1
K97A	12	134	0.87
D113A	6	1	233
T123A	4	233	1.50
L125A	5	383	0.73
V127A	4	62	5.65
R166A	8	52	3.37
D180A	10	1	140
N197A	1	25	56
F210A	4	77	4.55
R252A	43	17	1.92
S256A	16	13	6.73
N276A	46	225	0.14
T283A	13	55	1.96
Q337A	9	83	1.87
S365A	64	534	0.04
P369A	8	129	1.36
G459A	9	54	2.88
G471A	11	37	3.44
D474A	11	91	1.40

^aIC₉₀, antibody concentration yielding 90% neutralization efficiency.

^bSee the text for details.

TABLE 3. Neutralization of wildtype and select mutant pseudovirions of JR-CSF by mAbs CD4-IgG2 and 2G12.

Pseudovirus	IC ₉₀ (μg/ml) for mAb ^a :		Apparent affinity relative to wildtype gp120 _{JR-CSF} (%) for mAb:		Neutralization index for mAb:	
	CD4-IgG2	2G12	CD4-IgG2	2G12	CD4-IgG2	2G12
wildtype	13	6	100	100	1	1
D113A	5	3	9	169	28.9	1.18
D180A	1	14	31	108	41.9	0.40
N197A	1	6	3	318	433	0.31
S256A	20	6	47	108	1.38	0.93
S365A	6	9	23	1800	9.42	0.04
N276A	30	3	27	169	1.60	1.18

^aIC₉₀, antibody concentration yielding 90% neutralization efficiency.

TABLE 4. Binding affinities of a panel of anti-CD4bs mAbs to recombinant gp120_{JR-FL} containing multiple alanine substitutions.

Mutant ^a	Apparent antibody affinity (%) relative to wildtype gp120 _{JR-FL} for:						
	b12	b6	b3	CD4	F91	15e	F105
GDMR	250	0.1	0.1	0.1	82	63	0.1
DMR	167	0.1	0.1	0.1	64	112	0.1
DR	167	0.1	0.1	43	90	112	0.1
GM	120	9	0.1	0.1	75	68	0.1

^aMutants are denoted by the amino acids that were changed to alanine. G, G473; D, D474; M, M475; R, R476.

[0205] Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the claims, which follow the List of References.

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What is claimed is:

1. An immunogenic mutant HIV-1 gp120 polypeptide that can stimulate a neutralizing antibody response against a panel of human immunodeficiency virus type 1 (HIV-1) comprising HIV-1 primary isolates of at least two different clades, which mutant HIV-1 gp120 polypeptide has at least one amino acid mutation in at least one epitope of the HIV-1 gp120 polypeptide specifically bound by a non-neutralizing antibody, which mutation reduces binding affinity of the non-neutralizing antibody.
2. The immunogenic mutant HIV-1 gp120 polypeptide of claim 1, wherein said mutation increases the apparent binding affinity of a neutralizing antibody to at least 120% of the unmutated HIV-1 gp120 polypeptide.
3. The immunogenic mutant HIV-1 gp120 polypeptide of claim 1 or 2, wherein the panel of human immunodeficiency virus type 1 (HIV-1) comprises HIV-1 primary isolates of at least five different clades selected from clades A, B, C, D, E, F, and G.
4. The immunogenic mutant HIV-1 gp120 polypeptide of anyone of claims 1-3, wherein said mutation reduces the apparent binding affinity of the non-neutralizing antibody to 1% to 0.1% of the unmutated HIV-1 gp120 polypeptide.
5. The immunogenic mutant HIV-1 gp120 polypeptide of anyone of claims 1-4, wherein the unmutated HIV-1 gp120 polypeptide is derived from HIV-1 primary isolate JR-CSF or JR-FL.
6. An immunogenic mutant gp120 polypeptide that can stimulate a neutralizing antibody response against a human immunodeficiency virus (HIV), said mutant gp120 polypeptide comprising an HIV gp120 polypeptide having at least one amino acid mutation in at least one epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.

7. The immunogenic mutant gp120 polypeptide of claim 6, comprising an HIV gp120 polypeptide having at least two amino acid mutations in at least one epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
8. The immunogenic mutant gp120 polypeptide of claim 7, wherein the at least two amino acid mutations comprise at least a first amino acid mutation in a first epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody and at least a second amino acid mutation in a second epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
9. The immunogenic mutant gp120 polypeptide of claim 8, wherein the first epitope and the second epitope are different.
10. The immunogenic mutant gp120 polypeptide of claim 9, wherein the epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody comprises F43 cavity perimeter amino acid residues, V1 loop amino acid residues, V2 loop amino acid residues, V3 loop amino acids residues, non-neutralizing face amino acid residues, C1 domain amino acid residues, C5 domain amino acid residues, co-receptor binding site amino acid residues, or a combination thereof.
11. The immunogenic mutant gp120 of claim 6, wherein the at least one amino acid mutation is a substitution of a first amino acid residue for a second amino acid residues, wherein the second amino acid residue is different from the first amino acid residue.
12. The immunogenic mutant gp120 of claim 6, wherein the at least one amino acid mutation is a substitution of an amino acid residue of the gp120 polypeptide with an alanine.

13. The immunogenic mutant gp120 of claim 6, wherein the at least one amino acid mutation generates a glycosylation motif.
14. The immunogenic mutant gp120 of claim 13, wherein the glycosylation motif comprises an N-linked glycosylation motif.
15. The immunogenic mutant gp120 of claim 6, wherein the HIV is HIV type 1 (HIV-1).
16. The immunogenic mutant gp120 of claim 15, wherein at least one mutation comprises a substitution of G473, D474, M475, R476, or a combination thereof.
17. The immunogenic mutant gp120 of claim 15, wherein at least one mutation comprises a substitution of G473A, D474A, M475A, R476A, or a combination thereof.
18. The immunogenic mutant gp120 of claim 17, comprising at least two amino acid mutations.
19. The immunogenic mutant gp120 of claim 18, which comprises D474A and R476A; G473A and M475A; D474A, M475A and R476A; or G473A, D474A, M475A and R476A.
20. The immunogenic mutant gp120 of claim 15, comprising G473A, D474A, M475A and G476A.
21. The immunogenic mutant gp120 of claim 15, wherein the amino acid mutation comprises D113A, V127A, D180A, N197A, S256A, or a combination thereof.

22. The immunogenic mutant gp120 of claim 15, wherein at least one amino acid mutation generates a glycosylation motif.
23. The immunogenic mutant gp120 of claim 22, wherein the glycosylation motif comprises an N-linked glycosylation motif.
24. The immunogenic mutant gp120 of claim 23, wherein the at least one amino acid mutation comprises H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; or I423N and N425T; or a combination thereof.
25. The immunogenic mutant HIV-1 gp120 of anyone of the claims 1-5, which comprises the amino acid mutations D474A and R476A; and furthermore comprises at least one amino acid mutation selected from H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or any combination thereof.
26. The immunogenic mutant HIV-1 gp120 of anyone of the claims 1-5, which comprises the amino acid mutations G473A and M475A; and furthermore comprises at least one amino acid mutation selected from H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or any combination thereof.
27. The immunogenic mutant HIV-1 gp120 of anyone of the claims 1-5, which comprises the amino acid mutations D474A, M475A and R476A; and furthermore comprises at least one amino acid mutation selected from H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or any combination thereof.

28. The immunogenic mutant HIV-1 gp120 of anyone of the claims 1-5, which comprises the G473A, D474A, M475A and R476A; and furthermore comprises at least one amino acid mutation selected from H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or any combination thereof.
29. The immunogenic mutant gp120 polypeptide of claim 23, comprising Q114N, L116T, S143T, E150N, and G152T.
30. The immunogenic mutant gp120 polypeptide of claim 23, comprising H92N, N94T, K171N, Y173T, I423N and N425T.
31. The immunogenic mutant gp120 polypeptide of claim 29, further comprising G373A, D474A, M475A, and R476A.
32. The immunogenic mutant gp120 polypeptide of claim 19, further comprising H92N, N94T, Q114N, L116T, S143T, E150N, G152T, K171N, Y173T, Q246N, P313N, R315T, I423N and N425T.
33. The immunogenic mutant gp120 polypeptide of claim 10, which comprises a deletion of C1 domain amino acid residues, C5 domain residues, or a combination thereof.
34. A method of inducing antibodies that can neutralize human immunodeficiency virus type 1 (HIV-1), comprising immunizing a subject with an immunogenic mutant HIV-1 gp120 polypeptide that can stimulate a neutralizing antibody response against a panel of human immunodeficiency virus type 1 (HIV-1) comprising HIV-1 primary isolates of at least two different clades, which mutant comprises an HIV-1 gp120 polypeptide having at least one amino acid mutation in at least one epitope of the HIV-1 gp120 polypeptide specifically bound by a non-neutralizing antibody, which mutation reduces binding affinity of the non-neutralizing antibody.

35. The method of claim 34, wherein said mutation in the immunogenic mutant HIV-1 gp120 polypeptide increases the apparent binding affinity of a neutralizing antibody to at least 120% of the unmutated HIV-1 gp120 polypeptide.
36. The method of claims 34 and 35, wherein the immunogenic mutant HIV-1 gp120 polypeptide can stimulate a neutralizing antibody response against a panel of human immunodeficiency virus type 1 (HIV-1) comprising HIV-1 primary isolates of at least five different clades selected from clades A, B, C, D, E, F, and G.
37. The method of the claims 34-36, wherein said mutation in the immunogenic mutant HIV-1 gp120 polypeptide reduces the apparent binding affinity of the non-neutralizing antibody to 1% to 0.1% of the unmutated HIV-1 gp120 polypeptide.
38. The method of anyone of the claims 34-37, wherein the unmutated HIV-1 gp120 polypeptide parent of the immunogenic mutated HIV-1 gp120 polypeptide is derived from HIV-1 primary isolate JR-CSF or JR-FL.
39. A method of inducing antibodies that can neutralize human immunodeficiency virus (HIV), comprising immunizing a subject with an immunogenic mutant gp120 polypeptide that can stimulate a neutralizing antibody response against a human immunodeficiency virus (HIV), said mutant gp120 polypeptide comprising an HIV gp120 polypeptide having at least one amino acid mutation in at least one epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
40. The method of claim 39, wherein the epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody comprises F43 cavity perimeter amino acid residues, V1 loop amino acid residues, V2 loop amine acid residues, V3 loop amine acid residues, non-neutralizing face amine acid residues, C1 domain

amino acid residues, C5 domain amino acid residues, co-receptor binding site amino acid residues, or a combination thereof.

41. The method of claim 39, comprising at least two amino acid mutations in at least one epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
42. The method of claim 39, comprising at least two amino acid mutations in at least two epitopes of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
43. The method of claim 39, wherein the at least one amino acid mutation comprises at least one amino acid substitution.
44. The method of claim 43, wherein the at least one amino acid substitution generates a glycosylation motif.
45. The method of claim 44, wherein the glycosylation motif comprises an *N*-linked glycosylation motif.
46. The method of claim 39, wherein the HIV is HIV type 1 (HIV-1).
47. The method of claim 46, wherein the immunogenic mutant gp120 polypeptide comprises D113A; V127A; D180A; N197A; S256A; G473A; D474A; M475A; R476A; H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or a combination thereof.

48. The method of claim 46, wherein the immunogenic mutant gp120 polypeptide comprises D474A and R476A; G473A and M475A; D474A, M475A and R476A; or G473A, D474A, M475A and R476A.
49. The method of claim 46, wherein the immunogenic mutant gp120 polypeptide comprises H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; or I423N and N425T; or a combination thereof.
50. The method of claim 46, wherein the immunogenic mutant gp120 polypeptide further comprises G473A, D474A, M475A and R476A.
51. The method of claim 50, wherein the immunogenic mutant gp120 polypeptide further comprises H92N, N94T, Q114N, L116T, S143T, E150N, G152T, K171N, Y173T, Q246N, P313N, R315T, I423N and N425T.
52. The method of anyone of the claims 34-38, wherein the immunogenic mutant HIV-1 gp120 comprises the amino acid mutations D474A and R476A; and furthermore comprises at least one amino acid mutation selected from H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or any combination thereof.
53. The method of anyone of the claims 34-37, wherein the immunogenic mutant HIV-1 gp120 comprises the amino acid mutations G473A and M475M; and furthermore comprises at least one amino acid mutation selected from H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or any combination thereof.
54. The method of anyone of the claims 34-37, wherein the immunogenic mutant HIV-1 gp120 comprises the amino acid mutations D474A, M475A and R476A; and

furthermore comprises at least one amino acid mutation selected from H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or any combination thereof.

55. The method of anyone of the claims 34-37, wherein the immunogenic mutant HIV-1 gp120 comprises the amino acid mutations G473A, D474A, M475A and R476A; and furthermore comprises at least one amino acid mutation selected from H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or any combination thereof.
56. A vaccine comprising one or more mutant gp120 polypeptides of anyone of the claims 1-5 or 25-28.
57. The method of claim 50, wherein the immunogenic mutant gp120 polypeptide further comprises a deletion of C1 domain amino acid residues, C5 domain amino acid residues, or a combination thereof.
58. The method of claim 39, further comprising obtaining antiserum from the subject, said antiserum comprising HIV neutralizing antibodies.
59. The method of claim 58, wherein the antiserum comprises HIV-1 neutralizing antibodies.
60. The method of claim 60, wherein the subject is a human subject.
61. The method of claim 58, further comprising isolating an HIV neutralizing antibody fraction from said antiserum.
62. The method of claim 39, further comprising substantially purifying HIV neutralizing antibodies from the subject.

63. Antiserum obtained by the method of claim 58.
64. An isolated HIV neutralizing antibody fraction obtained by the method of claim 61.
65. A substantially purified HIV neutralizing antibody obtained by the method of claim 62.
66. The substantially purified HIV neutralizing antibody of claim 65, which comprises polyclonal HIV-1 neutralizing antibodies.
67. The substantially purified HIV neutralizing antibody of claim 65, which comprises a monoclonal HIV neutralizing antibody.
68. The substantially purified HIV neutralizing antibody of claim 65, which comprises a monoclonal HIV-1 neutralizing antibody.
69. A method of ameliorating a human immunodeficiency virus (HIV) infection in a subject, comprising administering an immunogenic mutant gp120 polypeptide that can stimulate a neutralizing antibody response against HIV to the subject, said mutant gp120 polypeptide comprising an HIV gp120 polypeptide having at least one amino acid mutation in at least one epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
70. The method of claim 69, wherein the HIV is an HIV type 1 (HIV -1).
71. A method of preventing HIV-1 infection or ameliorating HIV-1 infection in a human subject, comprising administering one or more mutant gp120 polypeptides of anyone of the claims 1-5 or 25-28.

72. The method of claim 69, wherein the epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody comprises F43 cavity perimeter amino acid residues, V1 loop amino acid residues, V2 loop amino acid residues, V3 loop amino acid residues, non-neutralizing face amino acid residues, C1 domain amino acid residues, C5 domain amino acid residues, co-receptor binding site amino acid residues, or a combination thereof.
73. The method of claim 70, wherein the immunogenic mutant gp120 polypeptide comprises D113A; V127A; D180A; N197A; S256A; G473A; D474A; M475A; R476A; H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or a combination thereof.
74. The method of claim 70, wherein the immunogenic mutant gp120 polypeptide comprises D474A and R476A; G473A and M475A; D474A, M475A and R476A; or G473A, D474A, M475A and R476A.
75. The method of claim 70, wherein the immunogenic mutant gp120 polypeptide comprises H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; or I423N and N425T; or a combination thereof.
76. The method of claim 70, wherein the immunogenic mutant gp120 polypeptide comprises G473A, D474A, M475A and R476A.
77. The method of claim 76, wherein the immunogenic mutant gp120 polypeptide further comprises H92N, N94T, Q114N, L116T, S143T, E150N, G152T, K171N, Y173T, Q246N, P313N, R315T, I423N, and N425T.

78. The method of claim 76, wherein the immunogenic mutant gp120 polypeptide further comprises a deletion of C1 domain amino acid residues, C5 domain amino acid residues, or a combination thereof.
79. A method of ameliorating a human immunodeficiency virus (HIV) infection in a subject, comprising administering HIV neutralizing antibodies to the subject, wherein the HIV neutralizing antibodies comprise antibodies stimulated in response to mutant gp120 polypeptide comprising an HIV gp120 polypeptide having at least one amino acid mutation in at least one epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
80. The method of claim 79, wherein the HIV is HIV type 1 (HIV-1).
81. A method of preventing HIV-1 infection or ameliorating HIV-1 infection in a human subject, comprising administering HIV-1 neutralizing antibodies to the subject, wherein the HIV-1 neutralizing antibodies comprise antibodies stimulated in response to the vaccine of claim 56.
82. The method of claim 79, wherein said HIV neutralizing antibodies are obtained by immunizing an individual with the immunogenic mutant gp120 polypeptide, and isolating the HIV neutralizing antibodies, or antiserum containing the antibodies, for the individual.
83. The method of claim 79, wherein said HIV neutralizing antibodies are obtained by immunizing an individual with the immunogenic mutant gp120 polypeptide, generating hybridomas from antibody producing cells from the individual following said immunizing, and isolating the HIV neutralizing antibodies from hybridomas expressing the HIV neutralizing antibodies.
84. The method of claim 83, wherein the antibody producing cells comprise spleen cells.

85. A method of reducing or preventing human immunodeficiency virus (HIV) infection in a subject, comprising administering an immunogenic mutant gp120 polypeptide that can stimulate a neutralizing antibody response against HIV to the subject, said mutant gp120 polypeptide comprising an HIV gp120 polypeptide having at least one amino acid mutation in at least one epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
86. The method of claim 85, wherein the HIV is HIV type 1 (HIV-1).
87. The method of claim 86, wherein the epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody comprises F43 cavity perimeter amino acid residues, V1 loop amino acid residues, V2 loop amino acid residues, V3 loop amino acid residues, non-neutralizing face amino acid residues, C1 domain amino acid residues, C5 domain amino acid residues, co-receptor binding site amino acid residues, or a combination thereof.
88. The method of claim 86, wherein the immunogenic mutant gp120 polypeptide comprises at least two amino acid mutations in at least one epitope of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
89. The method of claim 86, wherein the immunogenic mutant gp120 polypeptide comprises at least two amino acid mutations in at least two epitopes of the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody.
90. The method of claim 86, wherein the at least one amino acid mutation comprises an amino acid substitution that generates a glycosylation motif.
91. The method of claim 90, wherein the glycosylation motif comprises an *N*-linked glycosylation motif.

92. The method of claim 86, wherein the immunogenic mutant gp120 polypeptide comprises D113A, V127A, D180A, N197A; S256A; G473A; D474A; M475A; R476A; H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; I423N and N425T; or a combination thereof.
93. The method of claim 86, wherein the immunogenic mutant gp120 polypeptide comprises D474A and R476A; G473A and M475A; D474A, M475A and R476A; or G473A, D474A, M475A and R476A.
94. The method of claim 86, wherein the immunogenic mutant gp120 polypeptide comprises H92N and N94T; Q114N and L116T; S143T; E150N and G152T; K171N and Y173T; Q246N; P313N and R315T; or I423N and N425T; or a combination thereof.
95. The method of claim 86, wherein the immunogenic mutant gp120 polypeptide comprises G473A, D474A, M475A and R476A.
96. The method of claim 95, wherein the immunogenic mutant gp120 polypeptide further comprises H92N, N94T, Q114N, L116T, S143T, E150N, G152T, K171N, Y173T, Q246N, P313N, R315T, I423N, and N425T.
97. The method of claim 95, wherein the immunogenic mutant gp120 polypeptide further comprises a deletion of C1 domain amine acid residues, C5 domain amine acid residues, or a combination thereof.
98. A method of producing HIV neutralizing antibodies, comprising contacting antibody producing cells with an immunogenic mutant gp120 polypeptide comprising an HIV gp120 polypeptide having at least one amine acid mutation in at least one epitope of

the HIV gp120 polypeptide specifically bound by a non-neutralizing antibody, whereby the antibody producing cells are stimulated to produce HIV neutralizing antibodies.

99. The method of claim 98, wherein said contacting comprises contacting antibody producing cells in culture.
100. The method of claim 99, wherein the antibody producing cells comprise spleen cells.
101. The method of claim 99, further comprising isolating HIV neutralizing antibodies from the culture.
102. HIV neutralizing antibodies produced by the method of claim 98.
103. The HIV neutralizing antibodies of claim 102, which are HIV -1 neutralizing antibodies.
104. Isolated HIV neutralizing antibodies obtained by the method of claim 101.
105. The method of claim 98, wherein said contacting comprises administering the immunogenic mutant gp120 polypeptide to a subject under conditions sufficient for the immunogenic gp120 polypeptide to stimulate an immune response in the subject.
106. A method of eliciting HIV-1 neutralizing antibodies in a human antibody transgenic mouse by administering a vaccine comprising one or more mutant gp120 polypeptides of anyone of the claims 1-5 or 25-28.

107. Isolated HIV-1 neutralizing antibodies obtained by harvesting spleen and lymph nodes from the mouse immunized by the method of claim 106.
108. The method of claim 105, further comprising obtaining antiserum comprising the HIV neutralizing antibodies from the subject.
109. The method of claim 108, further comprising isolating an HIV neutralizing antibody fraction from the antiserum.
110. The method of claim 108, further comprising substantially purifying HIV neutralizing antibodies from the antiserum.
111. The method of claim 105, further comprising isolating antibody producing cells from the subject.
112. Antiserum obtained by the method of claim 108.
113. The antiserum of claim 112, which comprises HIV-1 neutralizing antibodies.
114. An isolated HIV neutralizing antibody fraction obtained by the method of claim 109.
115. The isolated HIV neutralizing antibody fraction antiserum of claim 114, which comprises HIV -1 neutralizing antibodies.
116. A substantially purified HIV neutralizing antibody obtained by the method of claim 110.
117. The substantially purified HIV neutralizing antibody of claim 116, which comprises an HIV -1 neutralizing antibody.

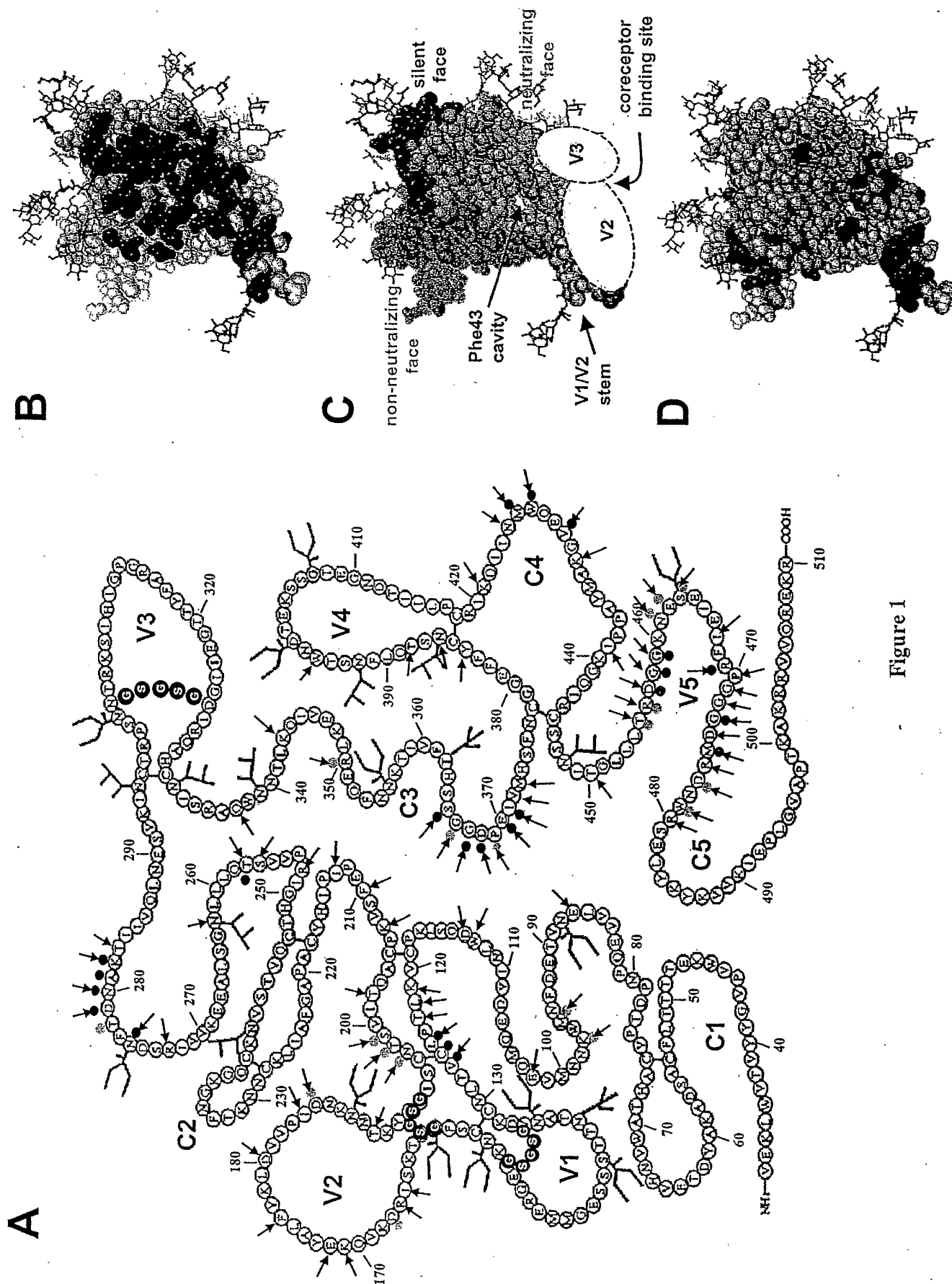


Figure 1

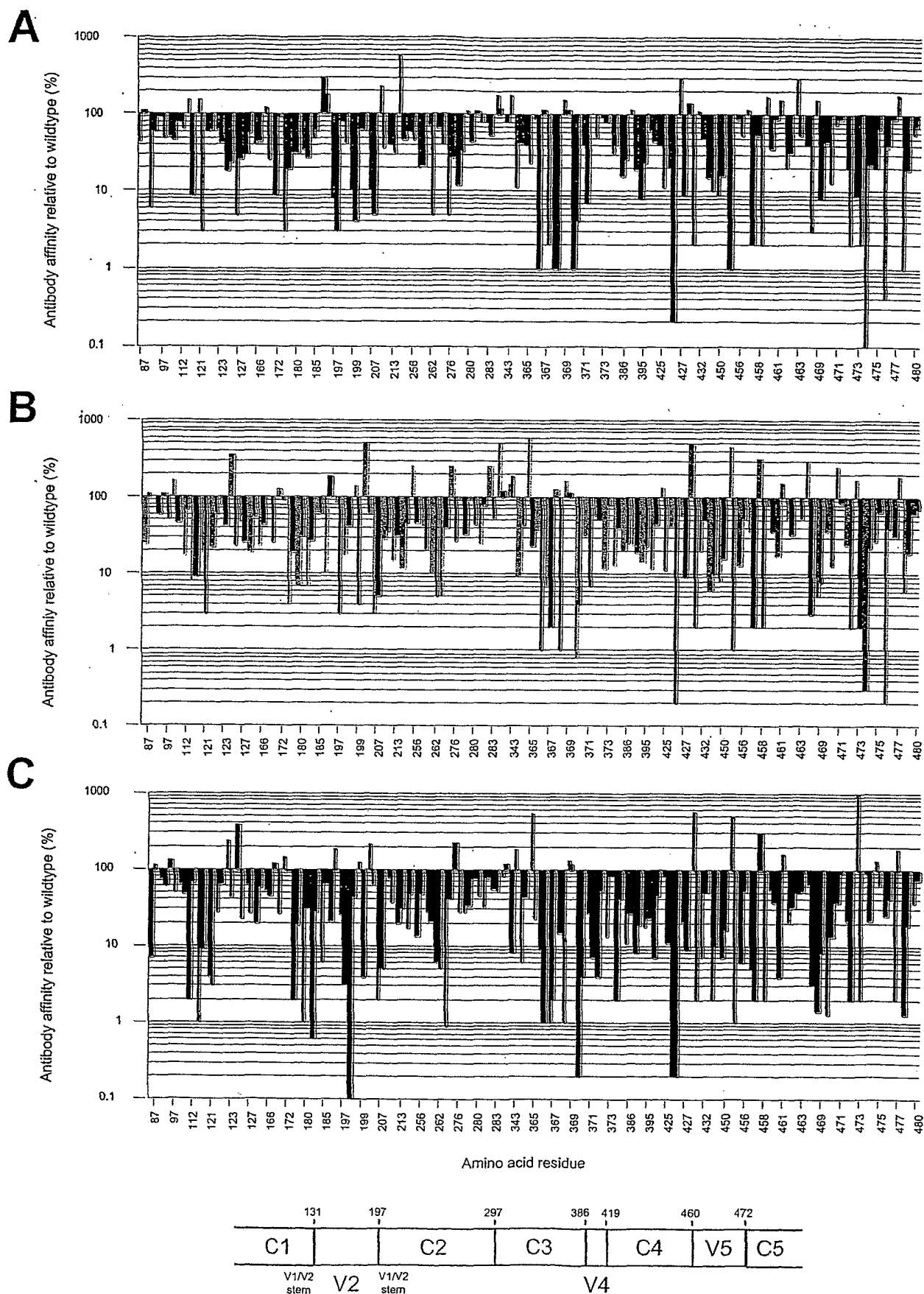


Figure 2

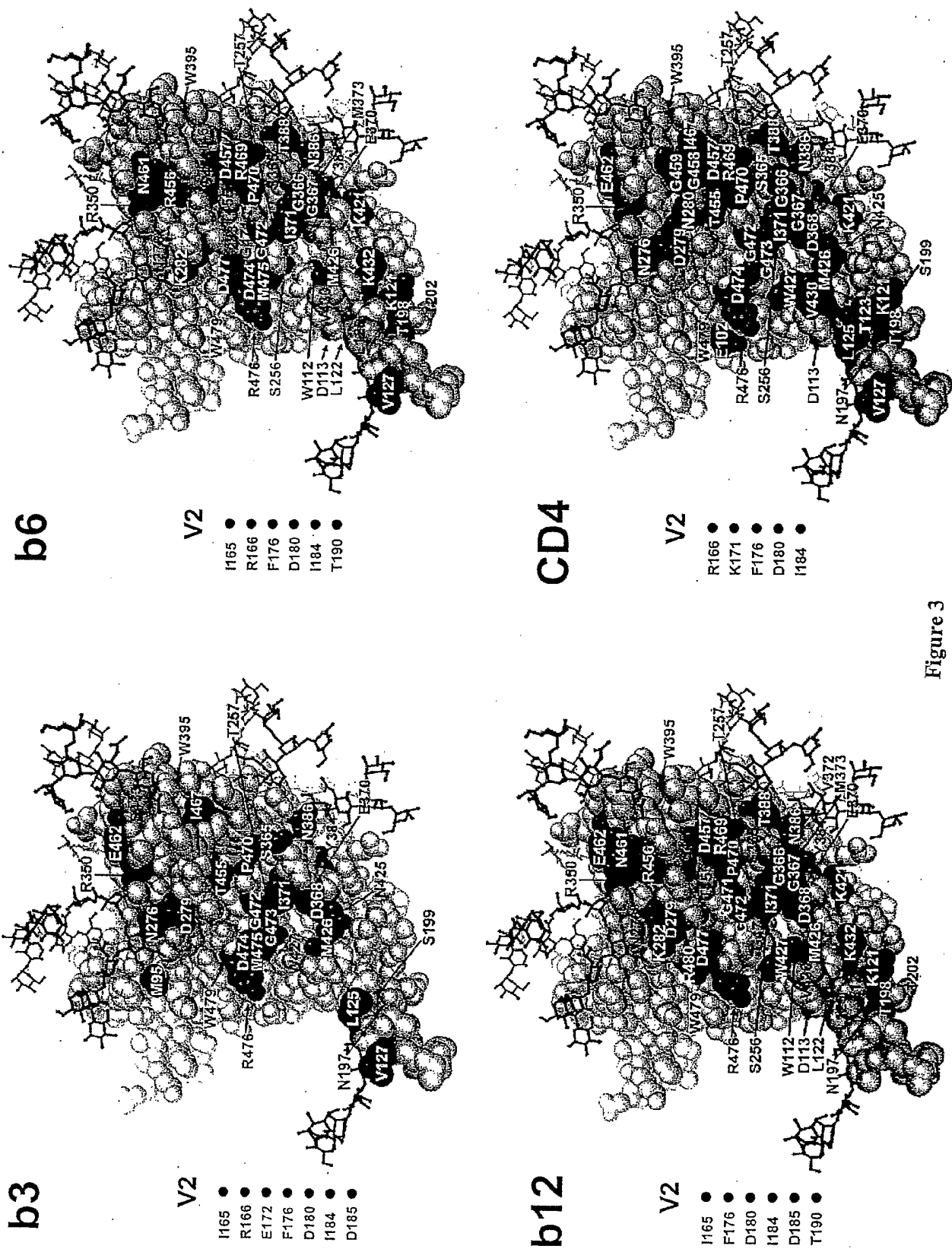
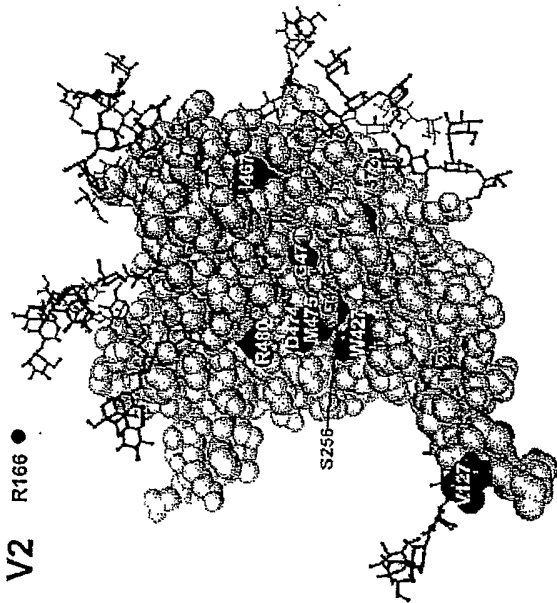


Figure 3

b12

V2

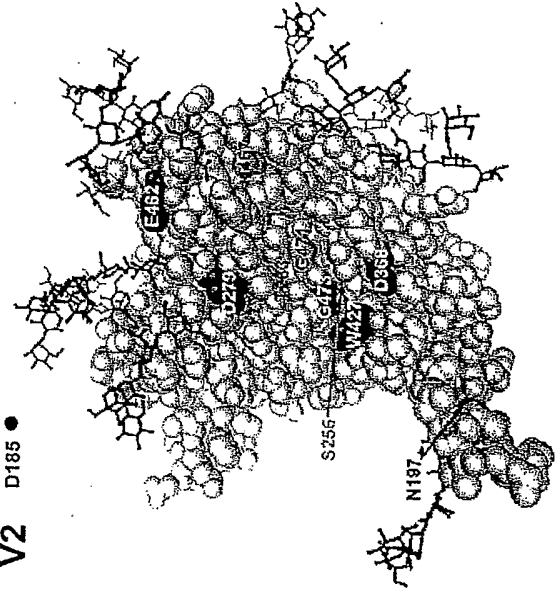
R166 ●



b6

V2

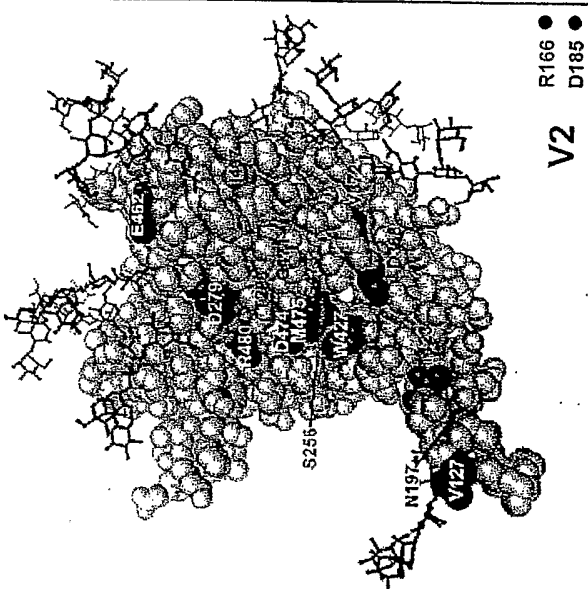
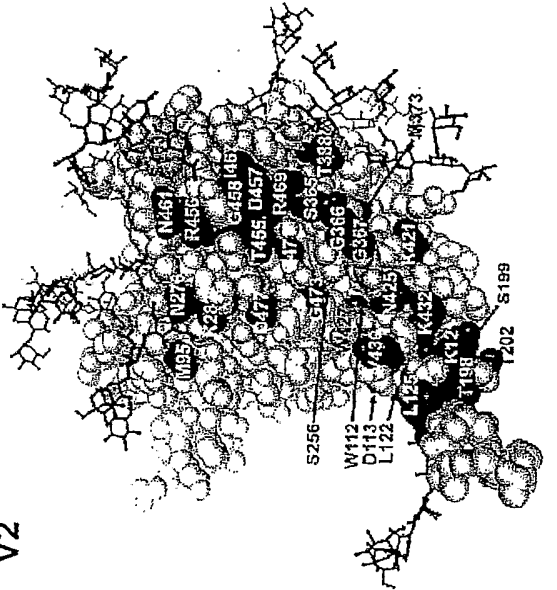
D185 ●



b3

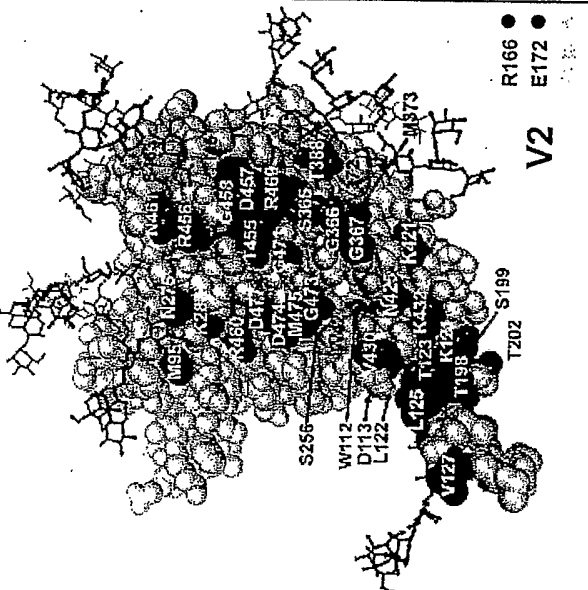
V2

E172 ●



V2

R166 ●
D185 ●



V2

R166 ●
E172 ●
D185 ●

Figure 4

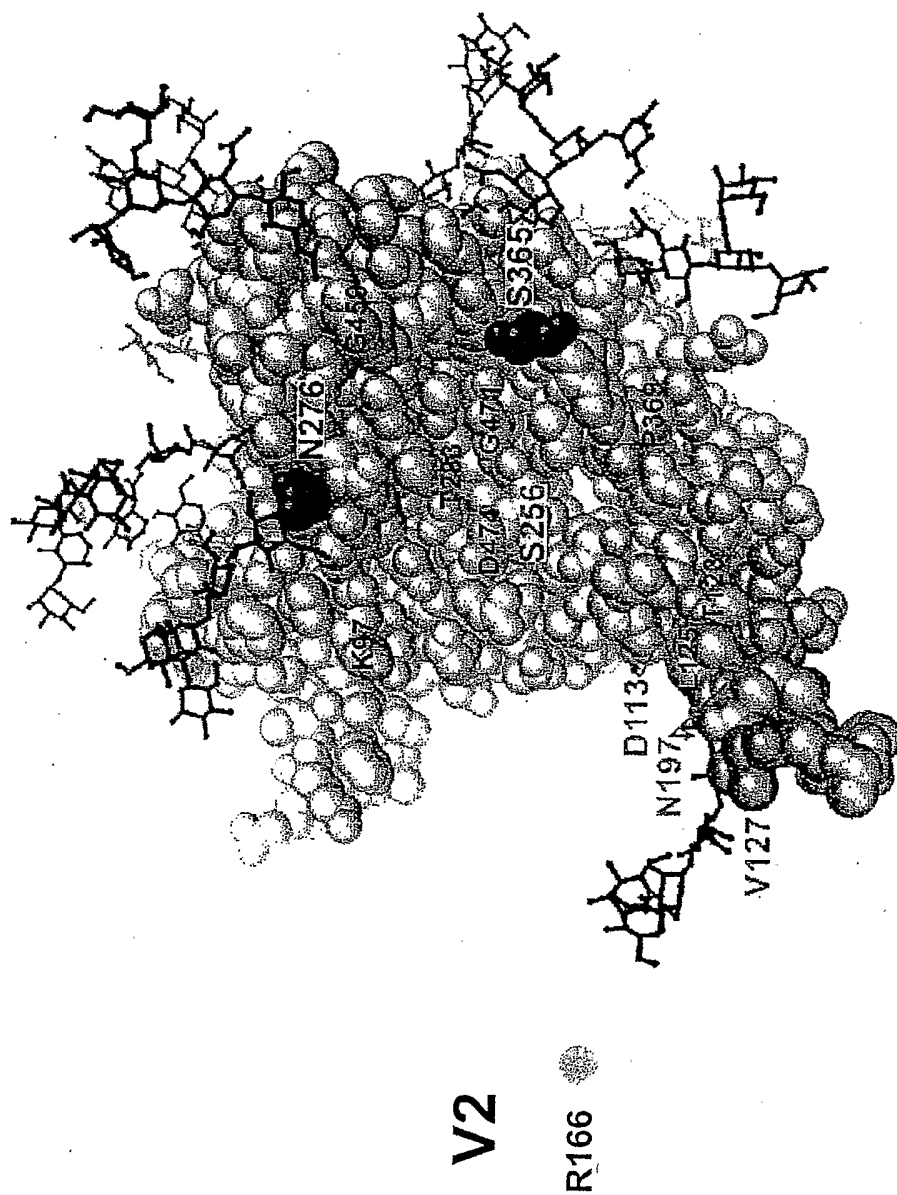


Figure 5

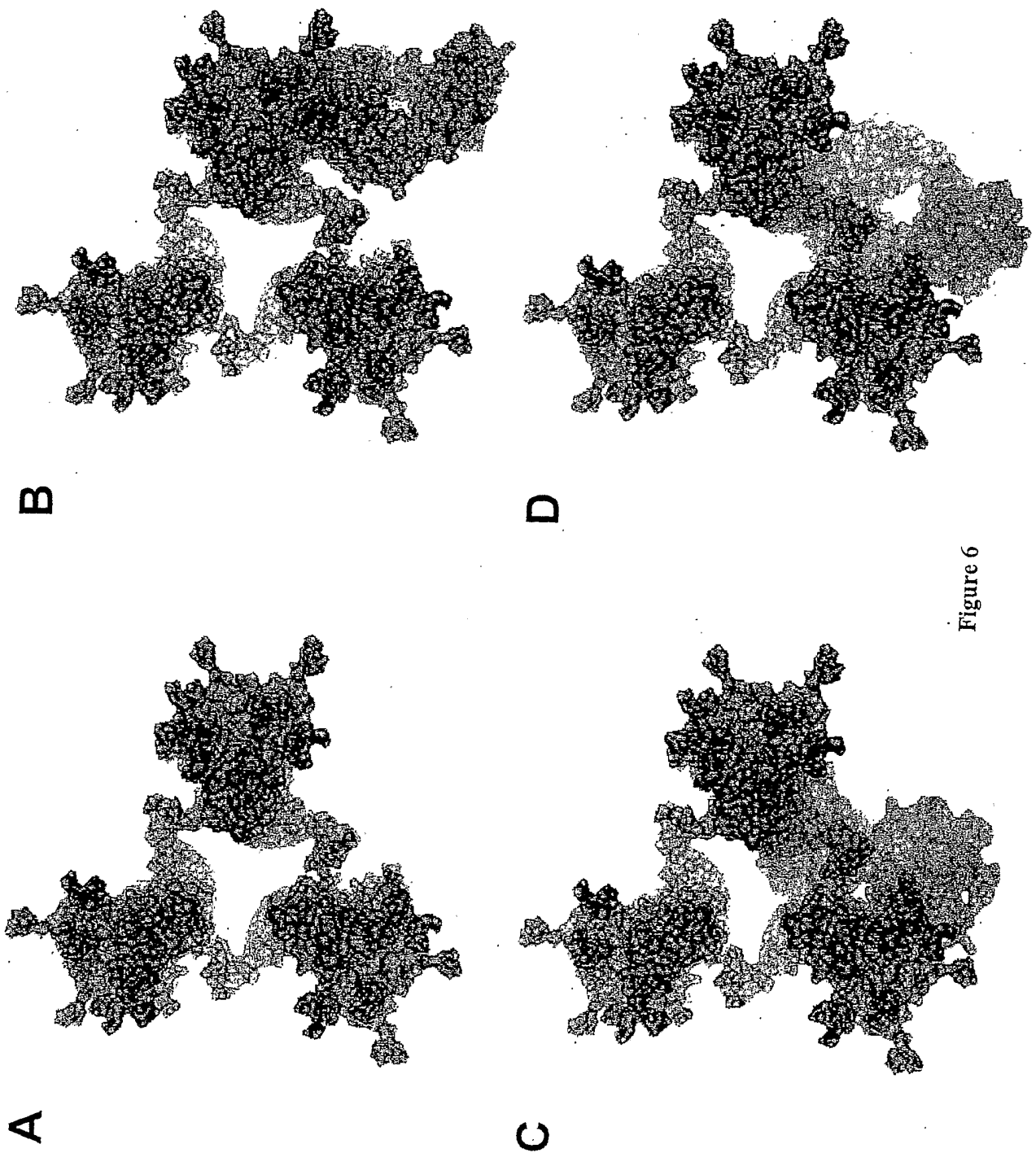


Figure 6

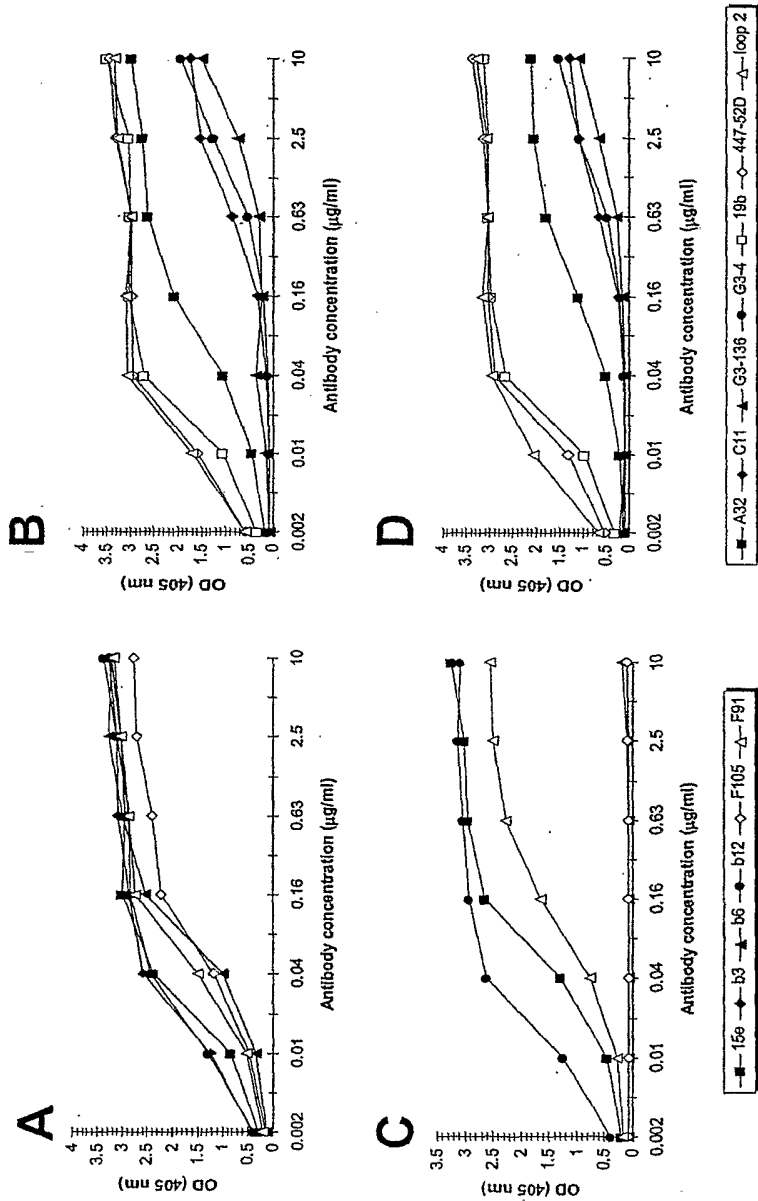


Figure 7

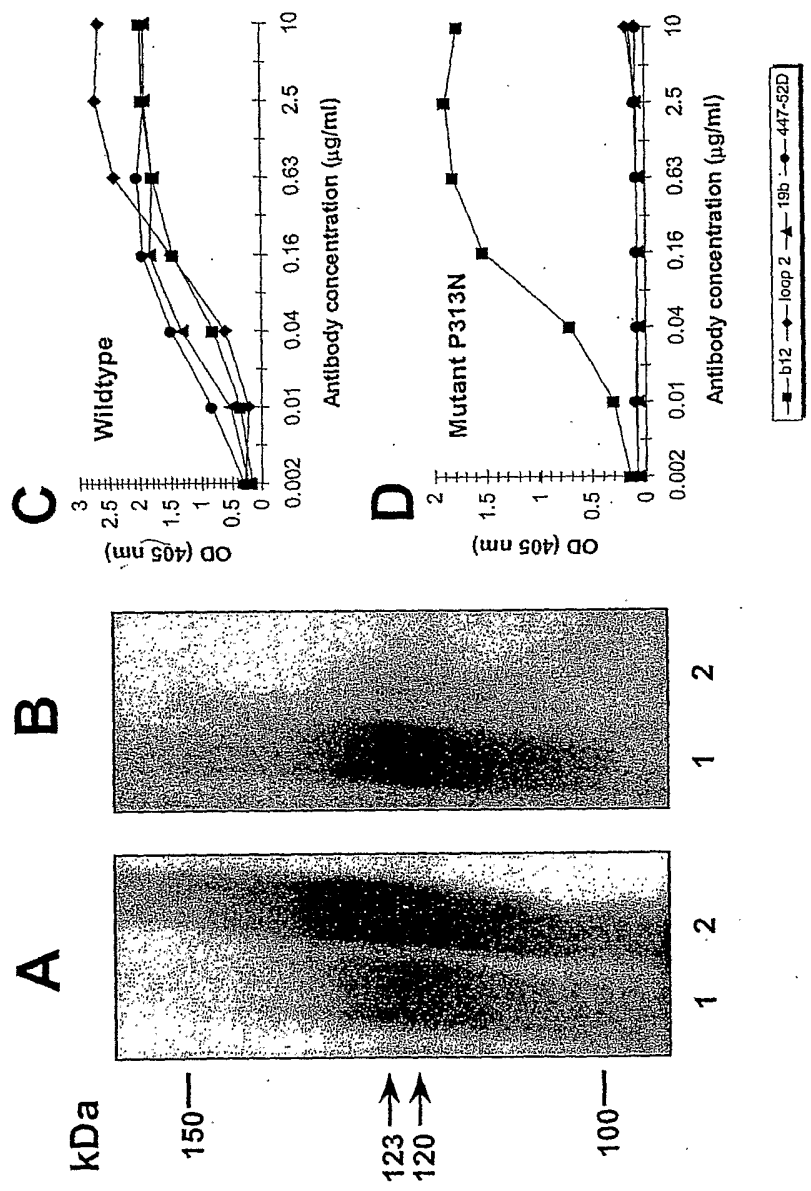


Figure 8

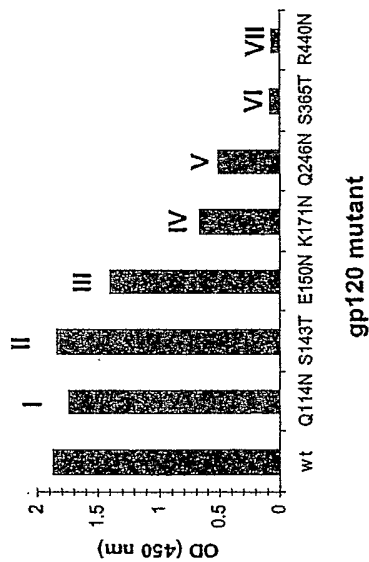


Figure 9

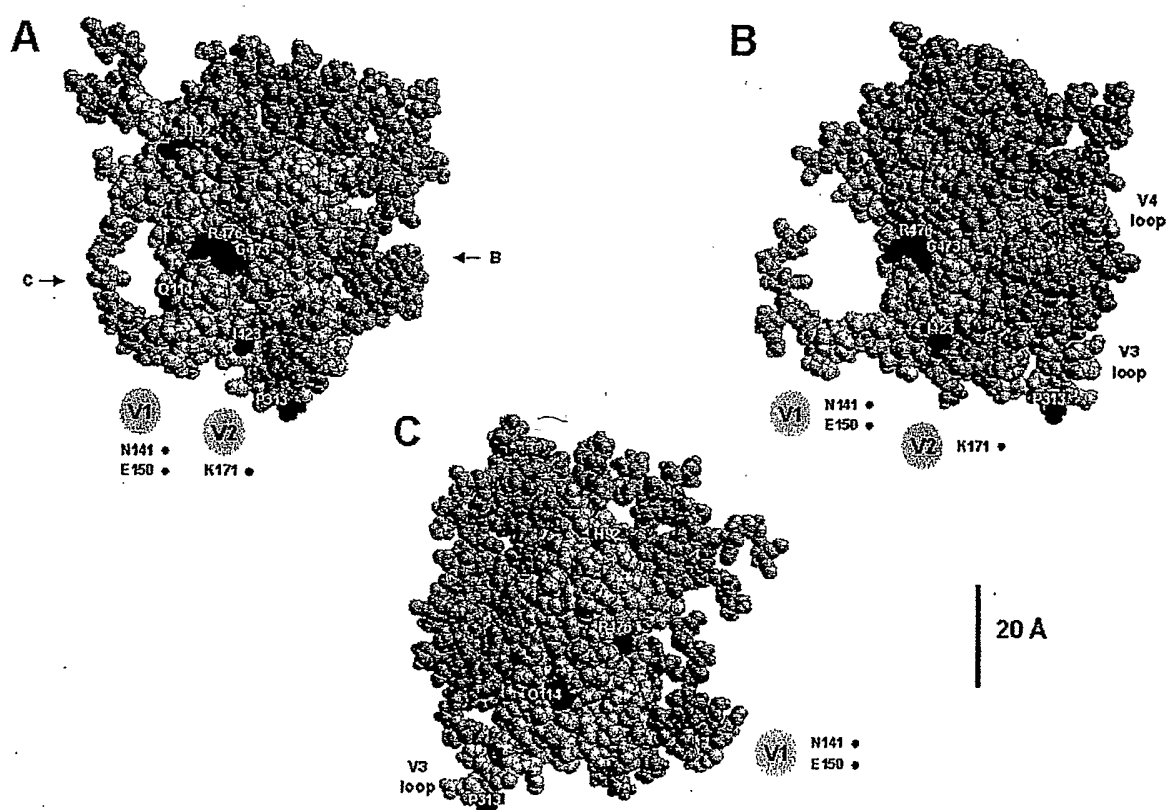


Figure 10

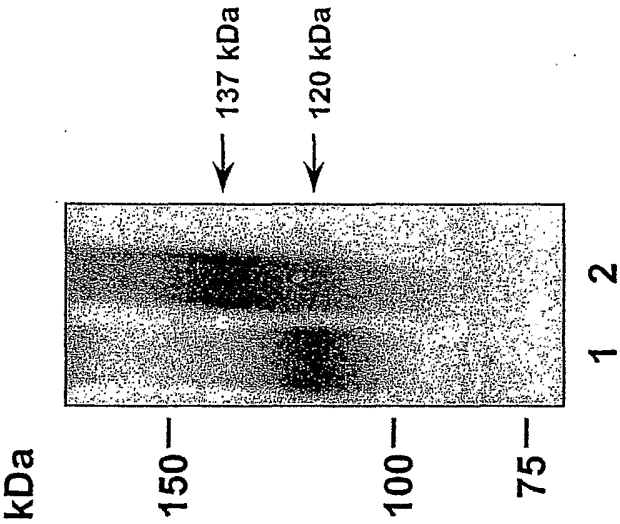


Figure 11

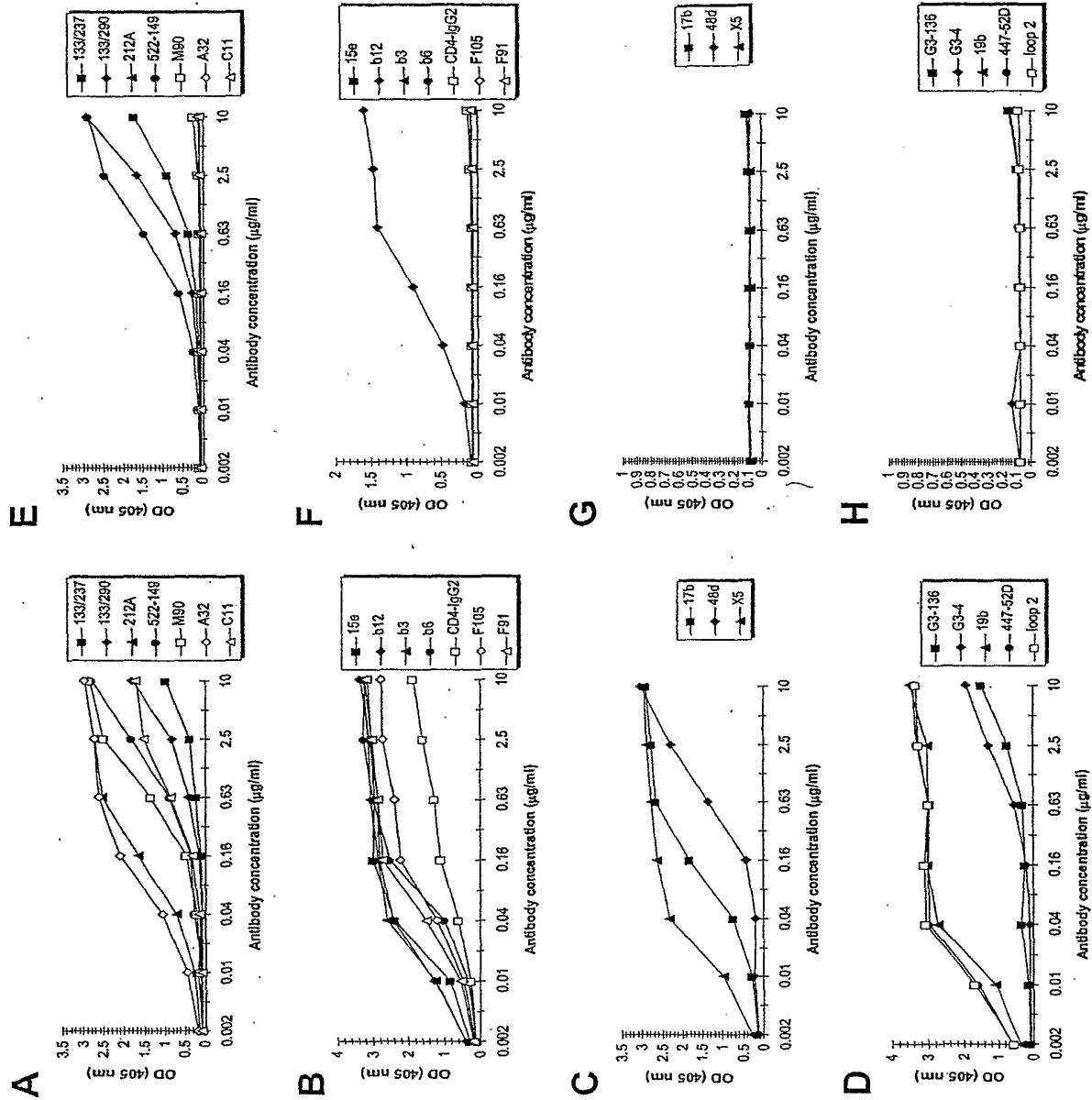


Figure 12

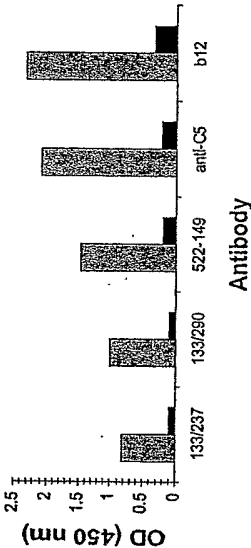


Figure 13

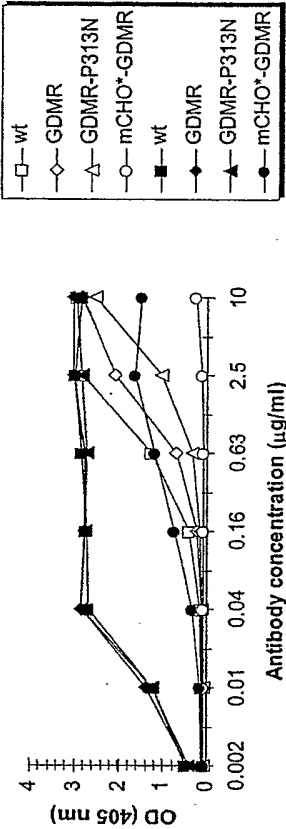


Figure 14

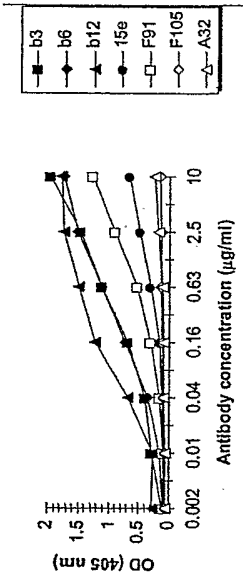


Figure 15

LOCUS AAB03749 848 aa linear VRL 12-JUL-1996
 DEFINITION envelope polyprotein.
 ACCESSION AAB03749
 VERSION AAB03749.1 GI:327819
 DBSOURCE locus HIVJRCSF accession M38429.1
 KEYWORDS .
 SOURCE Human immunodeficiency virus 1 (HIV-1)
 ORGANISM Human immunodeficiency virus 1
 Viruses; Retrovird viruses; Retroviridae; Lentivirus; Primate
 lentivirus group.
 REFERENCE 1 (residues 1 to 848)
 AUTHORS O'Brien,W.A., Koyanagi,Y., Namazie,A., Zhao,J.Q., Diagne,A.,
 Idler,K., Zack,J.A. and Chen,I.S.
 TITLE HIV-1 tropism for mononuclear phagocytes can be determined by
 regions of gp120 outside the CD4-binding domain
 JOURNAL Nature 348 (6296), 69-73 (1990)
 MEDLINE 91043044
 PUBMED 2172833
 COMMENT Kindly provided in computer-readable form by Irvin Chen, UCLA
 School of Medicine, Los Angeles. JRCSF and JRFL (see <HIVJRFL> were
 isolated from cerebral spinal fluid and brain tissue of the patient
 JR, who died with Kaposi's sarcoma and severe AIDS encephalo-
 pathy (Science 236, 819-822, 1987). Both clones are infectious, but JRFL
 productively infects macrophages while JRCSF does not. (Peripheral
 blood was not available from the patient).
 The JRCSF and JRFL env nucleotide sequences differ by at least 3%;
 further characterization of them is forthcoming (Peng,S. et al.,
 Nature 1990, in press). Both manifest insertions in nef previously
 reported for HIVBRVA.
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Appendix 1

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NCBI | NLM | NIH

Mar 17 2003 10:55:57

LOCUS HIVJRCSF 9540 bp ss-RNA linear VRL 12-JUL-1996
 DEFINITION Human immunodeficiency virus type 1, isolate JRCSF; complete genome.
 ACCESSION M38429
 VERSION M38429.1 GI:327813
 KEYWORDS long terminal repeat (LTR).
 SOURCE Human immunodeficiency virus 1 (HIV-1)
 ORGANISM Human immunodeficiency virus 1
 Viruses; Retrovird viruses; Retroviridae; Lentivirus; Primate lentivirus group.
 REFERENCE 1 (bases 1 to 9540)
 AUTHORS O'Brien, W.A., Koyanagi, Y., Namazie, A., Zhao, J.Q., Diagne, A., Idler, K., Zack, J.A. and Chen, I.S.
 TITLE HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain
 JOURNAL Nature 348 (6296), 69-73 (1990)
 MEDLINE 91043044
 PUBMED 2172833
 COMMENT Original source text: HIV-1 proviral DNA from extracellular virus taken from cerebral spinal fluid (1986). Infectious clone. Kindly provided in computer-readable form by Irvin Chen, UCLA School of Medicine, Los Angeles. JRCSF and JRFL (see <HIVJRFL> were isolated from cerebral spinal fluid and brain tissue of the patient JR, who died with Kaposi's sarcoma and severe AIDS encephalopathy (Science 236, 819-822, 1987). Both clones are infectious, but JRFL productively infects macrophages while JRCSF does not. (Peripheral blood was not available from the patient). The JRCSF and JRFL env nucleotide sequences differ by at least 3%; further characterization of them is forthcoming (Peng, S. et al., Nature 1990, in press). Both manifest insertions in nef previously reported for HIVBRVA.

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LOCUS AAB05604 847 aa linear VRL 02-AUG-1996
 DEFINITION envelope glycoprotein.
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 VERSION AAB05604.1 GI:1465781
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 KEYWORDS .
 SOURCE Human immunodeficiency virus 1 (HIV-1)
 ORGANISM Human immunodeficiency virus 1
 Viruses; Retroid viruses; Retroviridae; Lentivirus; Primate
 lentivirus group.
 REFERENCE 1 (residues 1 to 847)
 AUTHORS Koyanagi,Y., Miles,S., Mitsuyasu,R.T., Merrill,J.E., Vinters,H.V.
 and Chen,I.S.
 TITLE Dual infection of the central nervous system by AIDS viruses with
 distinct cellular tropisms
 JOURNAL Science 236 (4803), 819-822 (1987)
 MEDLINE 87206194
 PUBMED 3646751
 REFERENCE 2 (residues 1 to 847)
 AUTHORS O'Brien,W.A., Koyanagi,Y., Namazie,A., Zhao,J.Q., Diagne,A.,
 Idler,K., Zack,J.A. and Chen,I.S.
 TITLE HIV-1 tropism for mononuclear phagocytes can be determined by
 regions of gp120 outside the CD4-binding domain
 JOURNAL Nature 348 (6296), 69-73 (1990)
 MEDLINE 91043044
 PUBMED 2172833
 REFERENCE 3 (residues 1 to 847)
 AUTHORS Pang,S., Vinters,H.V., Akashi,T., O'Brien,W.A. and Chen,I.S.
 TITLE HIV-1 env sequence variation in brain tissue of patients with
 AIDS-related neurologic disease
 JOURNAL J. Acquir. Immune Defic. Syndr. 4 (11), 1082-1092 (1991)
 MEDLINE 92092169
 PUBMED 1684385
 REMARK Sequence fragments reported in this paper are corrections to
 sequence reported in first reference.
 REFERENCE 4 (residues 1 to 847)
 AUTHORS Pang,S., Vinters,H.V., Akashi,T., O'Brien,W.A., Chen,I.S.,
 Koyanagi,Y., Namazie,A., Zhao,J., Diagne,A. and Idler,K.
 TITLE Direct Submission
 JOURNAL Submitted (11-JUL-1996) T-10, Los Alamos National Lab, Mail Stop
 K710, Los Alamos, NM 87545, USA
 COMMENT JRFL AND JRCSF (See GenBank Accession Number M38492) were isolated
 from frontal lobe and cerebral spinal fluid of the patient JR, who
 died with Kaposi's sarcoma and severe AIDS encephalopathy (Science
 236, 819-822, 1987). Both clones are infectious, but JRFL
 productively infects macrophages while JRCSF does not (peripheral
 blood was not available from the patient). Both JRFL and JRCSF

isolates manifest insertions in nef previously reported for HIVBRVA. Sequences reported in GenBank Accession Numbers M74978-M75007 are other clones isolated from brain tissue of the same patient.

FEATURES Location/Qualifiers
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 /note="subtype B; infectious clone derived from provirus
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 /note="gp160 precursor is cleaved into gp120 and gp41."

ORIGIN

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LOCUS HIVU63632 8896 bp DNA linear VRL 03-AUG-1996
 DEFINITION HIV-1 isolate JRFL (USA) gag, pol, vif, vpr, tat, rev and env genes, complete cds.
 ACCESSION U63632
 VERSION U63632.1 GI:1465777
 KEYWORDS .
 SOURCE Human immunodeficiency virus 1 (HIV-1)
 ORGANISM Human immunodeficiency virus 1
 Viruses; Retroid viruses; Retroviridae; Lentivirus; Primate lentivirus group.
 REFERENCE 1 (bases 1 to 8896)
 AUTHORS Koyanagi,Y., Miles,S., Mitsuyasu,R.T., Merrill,J.E., Vinters,H.V. and Chen,I.S.
 TITLE Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms
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 MEDLINE 87206194
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 REFERENCE 2 (bases 1 to 8896)
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 TITLE HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain
 JOURNAL Nature 348. (6296), 69-73 (1990)
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 TITLE HIV-1 env sequence variation in brain tissue of patients with AIDS-related neurologic disease
 JOURNAL J. Acquir. Immune Defic. Syndr. 4 (11), 1082-1092 (1991)
 MEDLINE 92092169
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 REMARK Sequence fragments reported in this paper are corrections to sequence reported in first reference.
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 AUTHORS Pang,S., Vinters,H.V., Akashi,T., O'Brien,W.A., Chen,I.S., Koyanagi,Y., Namazie,A., Zhao,J., Diagne,A. and Idler,K.
 TITLE Direct Submission
 JOURNAL Submitted (11-JUL-1996) T-10, Los Alamos National Lab, Mail Stop K710, Los Alamos, NM 87545, USA
 COMMENT JRFL AND JRCSF (See GenBank Accession Number M38492) were isolated from frontal lobe and cerebral spinal fluid of the patient JR, who died with Kaposi's sarcoma and severe AIDS encephalopathy (Science 236, 819-822, 1987). Both clones are infectious, but JRFL productively infects macrophages while JRCSF does not (peripheral blood was not available from the patient). Both JRFL and JRCSF

isolates manifest insertions in nef previously reported for HIVBRVA. Sequences reported in GenBank Accession Numbers M74978-M75007 are other clones isolated from brain tissue of the same patient.

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