Abstract:
The present invention provides two HTV-2 peptides useful in the diagnosis of HTV-2 infection. The invention also provides a sensitive ELISA for the detection of HTV-2 infection and a method for discriminating between singly-infected with HIV-2 or dually-infected (HTV-I and HIV2) individuals.
Description

COMPOSITIONS AND METHODS FOR DIAGNOSING HIV-2 INFECTION

FIELD OF THE INVENTION

[0001] This invention relates to the field of infectious disease. In particular, this invention relates to human immunodeficiency virus type 2 (HIV-2) peptides and their use in the development of a diagnostic test for HIV-2 infection.

BACKGROUND OF THE INVENTION


[0003] Human immunodeficiency virus type 2 (HIV-2), the second AIDS virus isolated from West African subjects in 1985 [Clavel et al, Science 233:343-346 (1986)], is now present on all continents [Kanki et al, AIDS in Africa, 2nd ed. Kluer Academic/Plenum Publishers, New York, pp. 74-103 (2002); Kulkarni et al, Virology 337:68-75 (2005)]. The highest prevalence of HIV-2 in West Africa is found in Guinea-Bissau, where prevalence rates between 5-10 % of the adult urban population have been reported [van der Loeff et al, AIDS 13:S69-84 (1999)]. The highest prevalence of HIV-2 outside West Africa is found in Portugal where a prevalence rate of 3.4 % has been reported among AIDS cases [Comissao Nacional de Luta Contra a Sida, Documento SIDA 133/CVEDT (2004)].

[0004] There is limited knowledge on the natural history of HIV-2 infection because no study has investigated the full course of infection from the time of seroconversion. The available data suggest that people infected with HIV-2 have low levels of viremia and survive longer than individuals infected with HIV-1 [S. Andersson, AIDS Rev. 3:1 1-23 (2001); Barroso et al, AIDS Res. Hum. Retroviruses 20:1373-1376 (2004); Jaffar et al, Bull. World Health Organ. 82:462-
469 (2004)]. In subjects co-infected with HIV-I the disease may have a natural history similar to that of HIV-I [Jaffar et al, Bull. World Health Organ. 82:462-469 (2004)].

[0005] Likewise, little is known about the best approach to the clinical treatment of subjects infected with HIV-2. Many subjects infected with HIV-2 may not require treatment for a long period after infection [Jaffar et al, Bull. World Health Organ. 82:462-469 (2004)]. Subjects with declining CD4 cell counts may benefit from antiretroviral therapy but, given the slower development of immunodeficiency and persistent low viremia, it is unclear whether therapy will significantly slow progression [Houston et al, AIDS 16:1 189-1 191 (2002); Mullins et al, Clin. Infect. Dis. 38:1771-1779 (2004)]. Not all of the drugs used to treat HIV-I infection are as effective against HIV-2. In contrast to HIV-I, HIV-2 is naturally resistant to the (Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI) and may be less susceptible to Zidovudine (AZT) and some protease inhibitors (PIs), specially amprenavir and nelfinavir [Parkin et al, Antivir. Ther. 9:3-12 (2004); Reid et al, Virology 336:251-264 (2005); Witvrouw et al, Antivir. Ther. 9:57-65 (2004)].

[0006] Infection with HIV-I is usually associated with a vigorous humoral immune response to the gp120 and gp41 envelope glycoproteins and the core/matrix proteins p24 and pl7, encoded by the env and gag genes [J. Schupbach, Manual of Clinical Microbiology, 7th ed., American Society for Microbiology, pp. 847-870 (1999)]. In seroconvertors, the kinetics of the anti-gp41 and anti-gp20 antibody responses is similar, although anti-gp41 is usually detected earlier than anti-gp20 [Binley et al, J. Virol. 71:2799-2809 (1997); Parekh et al, AIDS Rev. 3:183-193 (2001); Zaaarjer et al, J. Med. Virol. 51:80-82 (1997)]. The antibody responses to the gp120 and gp41 proteins tend to be sustained throughout HIV-I infection, sometimes diminishing partially or completely only as AIDS-associated immunosuppression develop [Mullins et al, Clin. Infect. Dis. 38:1771-1779 (2004); J. Schupbach, Manual of Clinical Microbiology, 7th ed., American Society for Microbiology, pp. 847-870 (1999)]. The magnitude of the gp120 binding antibody response depends on the level of antigenic stimulation (viral load) and may be correlated with the immune functions and viremia control in chronically HIV-I infected subjects interrupting antiretroviral treatment [Trkola et al, Blood 104:1784-1792 (2004)]. Consistent with these observations, loss of antibody response to viral antigens (seroreversion) has occurred in HIV-I infected subjects following complete virologic suppression by antiretroviral therapy [Jurriaans et al, AIDS 18:1607-1608 (2004); Kassutto et al, Clin. Infect. Dis. 40:868-873 (2005)]. No studies has directly addressed these issues in HIV-2 infection. In the case of seronegative HIV-2 carriers recently described in India, it was suggested that extremely low production or non-
production of the antibody may have been caused by suboptimal immune stimulation due to very low HLV-2 replication [Kageyama et al, AIDS 11:31-37 (2000)]. Rare cases of seronegative HIV-1 infections have occurred in subjects with a rapid and aggressive disease course and were usually associated with immunological dysfunction [Cardoso et al, AIDS 18:1071-1074 (2004)].

[0007] Current diagnosis and surveillance strategies of HIV infection are based on the use of screening and confirmatory assays that detect antibodies produced against HIV-1 group M, N, O and HIV-2 [Gueye-Ndiaya, A., AIDS in Africa, 2nd ed., Kluer Academic/Plenum Publishers, New York, pp.121-138 (2002)]. Western blot or immunoblot tests are used solely for confirmation. The enzyme-linked immunosorbent assay (ELISA) is the most common test format used for the screening of HIV antibodies because it is highly sensitive and reproducible, simple to perform, and inexpensive. First generation HIV screening tests use viral lysate as antigen while second, third and fourth generation Ag-Ab combination assays use recombinant proteins and/or peptides as antigens on the solid phase [J. Schupbach, Manual of Clinical Microbiology, 7th ed., American Society for Microbiology, pp. 847-870 (1999)]. Most of the current serodiagnostic assays are mixed tests detecting anti-HIV-1 and anti-HIV-2 antibodies.

[0008] There remains a need in the art for an effective, highly sensitive, and highly specific rapid diagnostic test for HIV-2. There also remains a need for a simple, quick, cost-effective diagnostic test to differentiate between single infection with HIV-2 and co-infection with HIV-1 and HIV-2. The following invention provides a basis for such tests and significantly aims to fill the needs in this field.

**SUMMARY OF THE INVENTION**

[0009] By analyzing the immune response in a subject to certain proteins expressed by viruses such as HIV, an infection can be appropriately diagnosed and particular treatment regimes can be chosen and administered to the subject. The present invention is based, in part, upon the discovery that gp36 glycoprotein and the C2-C3 envelope protein are specific markers for HIV-2 infection, both of which generate an immune response in an infected subject. This discovery has been exploited to provide, at least in part, an invention that allows for the use of recombinant gp36 polypeptide and recombinant C2-C3 polypeptide to detect the presence of anti-gp36 and anti-C2-C3 antibodies in a subject. The presence of anti-gp36 antibodies and anti-C2-C3 antibodies is indicative of HIV-2 infection.

[0010] In one aspect, the invention provides a recombinant gp36 protein. The protein includes at least one ectopic polypeptide linked to a gp36 polypeptide or peptide fragment thereof in
which the gp36 polypeptide or peptide fragment thereof includes at least 30 amino acids from the amino acid sequence of SEQ ID NO: 1.

[0011] In certain embodiments, the ectopic polypeptide is selected from glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides. In particular embodiments, the ectopic polypeptide is at least one poly-histidine tag.

[0012] In other embodiments, the gp36 polypeptide includes at least 35 amino acids of SEQ ID NO: 1. In certain embodiments, the gp36 polypeptide includes at least 40 amino acids of SEQ ID NO: 1. In particular embodiments, the gp36 polypeptide includes at least 45 amino acids of SEQ ID NO: 1. In more particular embodiments, the gp36 polypeptide includes at least 50 amino acids of SEQ ID NO: 1. In still more particular embodiments, the gp36 polypeptide includes at least 55 amino acids of SEQ ID NO: 1. In yet more particular embodiments, the gp36 polypeptide is at least 95% homologous to the amino acid sequence from SEQ ID NO: 1.

[0013] In some embodiments, the recombinant gp36 protein also includes a peptide linker sequence linking the gp36 polypeptide or fragments thereof to at least one ectopic polypeptide.

[0014] In another aspect, the invention provides a polypeptide that includes at least 30 amino acids from the amino acid sequence of the C2-C3 envelope protein of HIV-2 represented as SEQ ID NO: 2. In some embodiments, the polypeptide is at least 35 amino acids of SEQ ID NO: 2. In certain embodiments, the polypeptide is at least 40 amino acids of SEQ ID NO: 2. In particular embodiments, the polypeptide is at least 45 amino acids of SEQ ID NO: 2. In more particular embodiments, the polypeptide is at least 50 amino acids of SEQ ID NO: 2. In yet more particular embodiments, the polypeptide is at least 95% homologous to the amino acid sequence of SEQ ID NO: 2.

[0015] In other embodiments, the polypeptide also includes at least one ectopic polypeptide linked to the C2-C3 polypeptide so as to form a recombinant C2-C3 protein. In certain embodiments, the ectopic polypeptide is selected from glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides. In particular embodiments, the ectopic polypeptide is at least one poly-histidine tag.

[0016] In some embodiments, the C2-C3 polypeptide includes at least 35 amino acids of SEQ ID NO: 2. In other embodiments, the C2-C3 polypeptide includes at least 40 amino acids of SEQ ID NO: 2. In certain embodiments, the C2-C3 polypeptide includes at least 45 amino acids of SEQ ID NO: 2. In particular embodiments, the C2-C3 polypeptide includes at least 50 amino acids of SEQ ID NO: 2. In more particular embodiments, the C2-C3 polypeptide includes at
least 55 amino acids of SEQ ID NO:2. In still more particular embodiments, the C2-C3 polypeptide is at least 95% homologous to the amino acid sequence from SEQ ID NO:2.

[0017] In some embodiments, the recombinant C2-C3 protein also includes a peptide linker sequence linking the C2-C3 polypeptide or fragments thereof to at least one ectopic polypeptide.

[0018] In yet another aspect, the invention provides a biological assay. The biological assay includes a recombinant gp36 protein and a recombinant C2-C3 protein immobilized on a solid substrate. In some embodiments, the recombinant gp36 protein includes at least one ectopic polypeptide linked to a gp36 polypeptide or fragment thereof from SEQ ID NO:1.

[0019] In other embodiments, the ectopic polypeptide is selected from glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides. In particular embodiments, the ectopic polypeptide includes a poly-histidine tag.

[0020] In some embodiments, the recombinant C2-C3 protein includes at least one ectopic polypeptide linked to a C2-C3 polypeptide or fragment thereof from SEQ ID NO:2. In certain embodiments, the ectopic polypeptide is selected from the group consisting of glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides. In particular embodiments, the ectopic polypeptide includes a poly-histidine tag.

[0021] In other embodiments, the recombinant gp36 protein is a poly-histidine-tagged gp36 polypeptide, the gp36 polypeptide being the amino-acid sequence of SEQ ID NO:1, and the recombinant C2-C3 protein is a poly-histidine-tagged C2-C3 polypeptide, the C2-C3 polypeptide being an amino acid sequence of SEQ ID NO:2. In certain embodiments, the solid substrate is selected from the group consisting of glass, polystyrene, PVDF membrane, nylon, and nitrocellulose.

[0022] In still another aspect, a method of detecting or diagnosing HIV-2 in a subject is provided. The method includes the step of contacting a recombinant gp36 protein and a recombinant C2-C3 protein with a fluid sample, which is isolated from a subject. The method then includes the step of specifically binding anti-gp36 antibodies and anti-C2-C3 antibodies that may be present in the fluid sample with the recombinant gp36 protein and the recombinant C2-C3 protein. The antibodies bound to the recombinant gp36 protein and the recombinant C2-C3 protein are then detected, which corresponds to the level of anti-gp36 and anti-C2-C3 antibodies in the fluid sample. If anti-gp36 antibodies and anti-C2-C3 antibodies are present and detected in the fluid sample, then HIV-2 is indicated.

[0023] In some embodiments, the presence of anti-gp36 antibodies and the presence of anti-C2-C3 antibodies are detected by secondary antibodies that specifically bind to human IgG or
human IgA. In certain embodiments, the secondary antibodies are labeled with a label selected from radiolabels, chemiluminescent labels, and fluorescent labels.

[0024] In other embodiments, the recombinant gp36 protein and the recombinant C2-C3 protein are immobilized on a solid substrate. In certain embodiments, the solid substrate is one of glass, polystyrene, PVDF membrane, nylon, and nitrocellulose.

[0025] In particular embodiments, the presence of anti-gp36 antibodies and anti-C2-C3 antibodies is detected by an enzyme-linked immunosorbent assay. In other embodiments, the presence of anti-gp36 antibodies and anti-C2-C3 antibodies is detected by immunoblot, dot blot, or protein microarray.

[0026] In still other embodiments, the method also includes the step of comparing the level of anti-C2-C3 antibodies detected in the fluid sample to a level of anti-C2-C3 antibodies detected in a control sample that is uninfected with HIV-2. In these embodiments, the presence of HIV-2 is indicated if the level of anti-C2-C3 antibodies detected in the fluid sample is greater than the level of anti-C2-C3 antibodies detected in the control sample.

[0027] In still another aspect, the invention provides a kit for diagnosing or detecting HIV-2 in a subject. The kit includes a recombinant gp36 protein, a recombinant C2-C3 protein, and a detection means to detect anti-gp36 and anti-C2-C3 antibodies. In some embodiments, the recombinant gp36 protein includes an ectopic polypeptide and a gp36 polypeptide or fragment thereof of SEQ ID NO:1.

[0028] In certain embodiments, the ectopic polypeptide is selected from glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides. In particular embodiments, the ectopic protein includes a poly-histidine tag.

[0029] In other embodiments, the recombinant C2-C3 protein includes at least one ectopic polypeptide linked to a C2-C3 polypeptide or fragment thereof from SEQ ID NO:2. In certain embodiments, the ectopic polypeptide is selected from glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides. In particular embodiments, the ectopic polypeptide includes a poly-histidine tag.

[0030] In particular embodiments, the recombinant gp36 protein is a poly-histidine-tagged gp36 polypeptide, the gp36 polypeptide being the amino acid sequence of SEQ ID NO:1, and the recombinant C2-C3 protein is a poly-histidine-tagged C2-C3 polypeptide, the C2-C3 polypeptide being an amino acid sequence of SEQ ID NO:2. In more particular embodiments, the recombinant gp36 protein and the recombinant C2-C3 protein are immobilized on a solid support. In still more particular embodiments, the solid substrate is selected from glass,
polystyrene, PVDF membrane, nylon, and nitrocellulose. In other embodiments, the detection means are labeled secondary antibodies that specifically bind to IgG and/or IgA.

[0031] In yet another aspect, a vaccine for treating or preventing a HIV-2 infection is provided. The vaccine includes a gp36 polypeptide, or peptide fragments thereof, and/or a C2-C3 polypeptide, or polypeptide subsequence thereof. The vaccine also includes at least one pharmaceutically acceptable vaccine component.

[0032] In some embodiments, the gp36 polypeptide or peptide fragments is a polypeptide sequence of SEQ ID NO:1. In other embodiments, the gp36 peptide fragments is at least ten amino acids long. In still other embodiments, the gp36 peptide fragments includes a hapten. In certain other embodiments, the C2-C3 polypeptide or peptide fragments is a polypeptide sequence of SEQ ID NO:2. In more embodiments, the C2-C3 peptide fragments is at least ten amino acids long. In some embodiments, the C2-C3 peptide fragments includes a hapten. In some embodiments, the pharmaceutically acceptable vaccine component is an adjuvant.

[0033] In yet another aspect, the invention provides a biological assay. The biological assay includes a recombinant gp36 protein consisting of a gp36 polypeptide linked to a poly-histidine-tag, the gp36 polypeptide being the amino acid sequence of SEQ ID NO: 1. The biological assay also includes a recombinant C2-C3 protein linked to a poly-histidine-tag, the C2-C3 polypeptide being an amino acid sequence of SEQ ID NO:2. The recombinant gp36 protein and the recombinant C2-C3 protein are immobilized on a solid support.

[0034] In still another aspect, the invention provides a method of detecting or diagnosing HIV-2 in a subject. The method includes a step of contacting a recombinant gp36 protein and a recombinant C2-C3 protein with a fluid sample isolated from a subject. The next step of the method includes allowing the anti-gp36 antibodies and anti-C2-C3 antibodies that are present in the fluid sample to specifically bind with the recombinant gp36 protein and the recombinant C2-C3 protein. The presence of anti-gp36 antibodies and anti-C2-C3 antibodies in the fluid sample is then detected. In addition, the method includes the step of comparing the level of anti-C2-C3

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0035] Figure IA is a graphic representation of the reactivity of HIV-I and HIV-2 plasma to rgp36 antigen as determined by S/CO [optical density of the sample (OD$_{\text{sample}}$) divided by the OD$_{\text{cut-off}}$ (optical density of HIV-seronegative samples + 3 standard deviations)].

[0036] Figure IB is a graphic representation of the reactivity of HIV-I and HIV-2 plasma to rpC2-C3 antigen as determined by S/CO.
[0037] Figure 1C is a graphic representation of the reactivity of HIV-2 plasma to rgp36 and rpC2-C3 as determined by S/CO.

[0038] Figure 1D is a graphic representation of low responders (LR) and high responders (HR) to rpC2-C3 antigen as determined by S/CO.

[0039] Figure 2 is a graphic representation of the Spearman rank correlation statistical test comparing the S/CO of rpC2-C3 to S/CO of rgp36.

**DETAILED DESCRIPTION OF THE INVENTION**

[0040] The present invention provides HIV-2 peptides useful in the development of an HIV-2 ELISA. The invention also provides a method to differentiate HIV-1 and HIV-2 in co-infected individuals. Further, the invention may provide a method to discriminate acute versus chronic infection and discern the timeline of infection HIV-2 positive subjects.

[0041] More specifically, the present invention is based, in part, upon the discovery that gp36 glycoprotein and the C2-C3 envelope protein are specific markers for HIV-2 infection, both of which generate an immune response in an infected subject. This discovery has been exploited to provide, at least in part, an invention that allows for the use of recombinant gp36 polypeptide and recombinant C2-C3 polypeptide to detect the presence of anti-gp36 and anti-C2-C3 antibodies in a subject. The presence of anti-gp36 antibodies and anti-C2-C3 antibodies is indicative of HIV-2 infection.

[0042] As used herein, the term "ectopic polypeptide" refers to a sequence of at least 5 or more amino acids that is fused to a particular protein or polypeptide sequence, but that is not associated, fused, linked, or covalently bound to the particular protein or polypeptide sequence as that protein or polypeptide sequence is found naturally.

[0043] As used herein, the term "recombinant" refers to an artificial sequence resulting from the combining of two other DNA sequences in a plasmid. Recombinant proteins are proteins that are produced by different genetically modified organisms following insertion of the relevant DNA into their genome.

[0044] As used herein, the term "linked" refers to the combination of two or more genetic sequences or two or more protein sequences so that the resulting genetic product or protein product will be a combination of the individual genes or proteins.

[0045] As used herein, the term "specifically bind(s)" refers to the interaction between an antigen or epitope and its receptor or between two complimentary proteins.

[0046] As used herein, the term "HIV-2" refers to the human immunodeficiency virus type 2 which is the second of two serotype for HIV. It is closely related to HIV-I and has also been
found to cause AIDS. Although HIV-I and HIV-2 are similar in their viral structure, modes of transmission, and disease manifestation, HIV-2 is less aggressive than HIV-I and has a marked lower sensitivity to antiretroviral pharmaceuticals compared to HIV-I.

The present invention also provides a means to differentiate HIV-I and HIV-2 in co-infected individuals. Dual HIV-I and HIV-2 seroreactivity is relatively frequent in countries where both HIV-I and HIV-2 are endemic such as Portugal (1.4%), Guinea-Bissau (0.7%), Senegal (0.4%) and India (up to 2%) [Holmgren et al, AIDS 17:241-253 (2003); Laurent et al, AIDS 17:1811-1816 (2003); Comisso Nacional de Luta Contra a Sida, Documento SIDA 133/CVEDT (2004); Paranjape et al, Indian J. Med. Res. 106:207-211 (1997)]. However, the true rate of dual infections in these countries is generally unknown. This is in part due to the lack of sensitive and specific HIV-2 antibody tests. In fact, only two ELISA tests of low specificity (92%) are currently available for the diagnosis of HIV-2 infection, both of which use the same viral lysate antigen [Azevedo-Pereira et al, J. Clin. Microbiol. 32:2559-2563 (1994); Center for Disease Control, MMWR 39:829-831 (1990)]. Most often, reactivity with gp36 or gpl25 derived antigens (peptides or recombinant proteins) incorporated into Western blot and immunoblot assays is used to distinguish between HIV-2 and HIV-I infections (J. Schupbach, Manual of Clinical Microbiology, 7th ed., American Society for Microbiology, pp. 847-870 (1999)). However, the sensitivity of these tests is generally low and serological cross-reactivity between the HIV-I and HIV-2 envelope glycoproteins has been described which may complicate the final diagnosis [Decker et al, J. Exp. Med. 201:1407-1419 (2005); Robert-Guroff et al, J. Virol. 66:3602-3608 (1992); Weiss et al, AIDS 2:95-100 (1998)].

Currently there are only two licensed enzyme immunoassay (EIA) assays for the specific serological diagnosis of HIV-2 infection [Center for Disease Control, MMWR 39:829-831 (1990)]. Both assays use whole-viral lysate as antigen. All other licensed kits use antigenic mixtures of recombinant proteins and/or synthetic peptides from HIV-I and HIV-2 enabling the combined detection of antibodies to both viruses.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>C2-C3</td>
<td>FRYCAPPGYALLRCDDINYSGFAPNC5KVAVAATCRMETQFTSTWGFNGTRAENRTYIYWHGRDNRITISSLNKHYNLTMICRPGNKTVPITLMMSFLFHPQI5PKRPQAWSFCWFKGEWRKAMQEVKETLVKYRKGDNTECINFTKPGDRGSADAEXVYMWT [SEQ ID NO: 2]</td>
</tr>
<tr>
<td>gp36</td>
<td>GTAALTLASQSRILLGIVQQQQQLLDVVKRQQMVLRLTVGTVKNIQARVTAIEKYLKDQARLNSWGCARQVCHTTPWVNNSLKPDWDNMTWQEWEQQVRYLEANISQLERAQIQKEDNTYELQKL [SEQ ID NO: 1]</td>
</tr>
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</table>
The present invention discloses an assay, ELISA-HIV2, which enables the independent detection and characterization of the antibody response of HIV-2 subjects to the recombinant proteins, rpC2-C3 and rgp36, derived respectively from the gp125 and the gp36 ectodomain of the envelope glycoprotein of the primary isolate HIV-2ALL. The clinical specificity of the assay was assessed with seronegative samples. There was no reactivity to the rpC2-C3 and rgp36 polypeptides. The 100% specificity of the ELISA-HIV2 assay compares favorably to the specificity of the two licensed HIV-2 serological diagnosis assays (<92%) [Azevedo-Pereira et al, J. Clin. Microbiol. 32:2559-2563 (1994); Center for Disease Control, MMWR 39:829-831 (1990)] and to the specificity of most mixed HIV-1/HIV-2 assays (mean 99%, range 94.6%-100% for assays based on recombinant proteins; mean 98%, range 90.4%-100% for assays based on synthetic peptides) [Andersson et al, AIDS 11:1815-1822 (1997); Beelaert et al, J. Virol. Methods 105:197-206 (2002); GalH et al, J. Clin. Microbiol. 34:999-1002 (1997)].

In the current invention, using a panel of HIV-2 samples, 100% clinical sensitivity was obtained with the rgp36 peptide alone. The finding that 93.4% of the HIV-2 samples reacted also with the rpC2-C3 peptide contrasts with the low immunoreactivity (below 81%) reported for recombinant proteins encoded by corresponding sequences in HIV-2 SBL6669 [Bottiger et al, J. Virol. 64:3492-3499 (1990)], HIV-2 ROD [Schulz et al, AIDS 3:165-172 (1989)], HIV-2NIHZ [Zuber et al, AIDS Res. Hum. Retroviruses 6:525-534 (1990)] and HIV-2 ST [Huang et al, J. Virol. 65:5073-5079 (1991)]. One explanation for this discrepancy is that rpC2-C3 may comprise epitopes which are more antigenic than the corresponding regions in HIV-2 strains SBL6669, ROD, NIHZ and ST, all of which are laboratory-adapted isolates. Therefore, antibodies present in the infected immune sera may recognize the HIV-2 ALI antigen better.

In Portugal, the reported rate of dual HIV-1/HIV-2 seropositivity is 1.4% but the true rate of dual infections is unknown [Comissao Nacional de Luta Contra a Sida, Documento SIDA 133/CVEDT (2004)]. With the ELISA-HIV2 assay described herein, none of seven dually seroreactive subjects were dually infected with HIV-1 and HIV-2. These results suggest that dual HIV-1/HIV-2 infections are rare in Portugal. Earlier reports suggested that most dually seropositive subjects from Guinea-Bissau (78-86%) [Walther-Jallow et al, AIDS Res. Hum. Retroviruses 15:957-962 (1999)], Ivory Coast and The Gambia (72%) [Ishikawa et al, AIDS 12:1419-1425 (1998)] were indeed dually infected. In more recent studies performed in India [Kannangai et al, J. Clin. Virol. 22:41-46 (2001)] and Senegal [Gottlieb et al, AIDS Res. Hum. Retroviruses 19:575-584 (2003)] a 40% rate of dual infections was reported among dually seroreactive subjects. Although the number of subjects is small in all studies, the declining
prevalence of dual infections that is documented is consistent with the worldwide decreasing incidence and prevalence rates of HIV-2 infection [Jaffar et al, Bull. World Health Organ. 82:462-469 (2004); Comisso National de Luta Contra a Sida, Documento SIDA 133/CVEDT (2004)].

According to the World Health Organization, the criteria used for Western Blot confirmation of HIV infection is reactivity against two envelope glycoproteins [Gueye-Ndiaya, A., AIDS in Africa, 2nd ed. Kluer Academic/Plenum Publishers, New York, pp.121-138 (2002)]. The ELISA-HIV2 assay as described in this invention was a competent confirmatory assay for HIV-2 infection since all Western Blot positive samples were also positive in ELISA-HIV2. Moreover, all five indeterminate samples in the Western Blot assay were HIV-2 negative in the ELISA-HIV2 assay. One indeterminate sample was dually HIV-1/HIV-2 seroreactive in Peptilav 1-2. Therefore, the ELISA-HIV2 assay is an excellent alternative to the confirmatory HIV-2 Western Blot assays.

Also, significant positive correlation was detected in the antibody responses of HIV-2 subjects to the two envelope proteins that were tested. On average, the antibody reactivity to the peptide rgp36 was two times stronger compared to rpC2-C3 peptide. Moreover, HIV-2 subjects could be clustered into two groups according to their antibody level to rpC2-C3 peptide. In the case for HIV-I, conflicting reports exist on the prognostic value of gpl20 antibody responses. Nevertheless, high gpl20 binding antibody titers were negatively correlated to immune functions and viremia control in chronically HIV-I infected subjects [Trkola et al, Blood 104:1784-1792 (2004)]. It will therefore be important to investigate the correlations between the titer of C2-C3 binding antibodies, viremia and immune functions, including neutralizing antibody response, in HIV-2 infection.

Antibodies to the envelope gp41 develop early in HIV-I infection while antibodies to the V3 region of gpl20 develop later in infection [Parekh et al, AIDS Rev. 3:183-193 (2001)]. Therefore, the different antibody responses to rpC2-C3 may be due to the timing of infection. Further testing of longitudinal specimens from seroconvertors will permit one of ordinary skill in the art to study the kinetics of antibody responses to this envelope protein and to assess the usefulness of this information to date the timing of HIV-2 infection.

**Peptide Modification and Purification**

To maximize the use of the peptides from the current invention, several different embodiments, modifications and variations may be used. The peptide may contain amino acids with charged side chains, such as acidic and basic amino acids. In addition, these peptides may
contain one or more D-amino acid residues in place of one or more L-amino acid residues provided that the incorporation of the one or more D-amino acids does not abolish all or so much of the activity of the peptide that it cannot be used in the compositions and methods of the invention. Incorporating D-amino acids in place of L-amino acids is favorable as it may provide additional stability to a peptide.

[0057] In another embodiment of the invention, the peptides may be capped at the amino or carboxy terminus. Preferred amino terminal capping groups include a lipoic acid moiety, which can be attached by an amide linkage to the ε-amino group of the amino terminus of a peptide. Another example of an amino terminal capping group useful in the peptides described herein is an acyl group, which can be attached in an amide linkage to the α-amino group of the amino terminal amino acid residue of a peptide.

[0058] In addition, in certain cases the amino terminal capping group may be a lysine residue or a polylysine peptide, where the polylysine peptide consists of two, three, or four lysine residues, which can prevent cyclization, crosslinking, or polymerization of the peptide compound. Alternatively, longer polylysine peptides may also be used. Another amino capping group that may be used in the peptides described in the invention is an arginine residue or a polyarginine peptide, where the polyarginine peptide consists of two, three, or four arginine residues, although longer polyarginine peptides may also be used. Alternatively the peptide compounds described herein may also be a peptide containing both lysine and arginine, where the lysine and arginine containing peptide is two, three, or four residue combinations of the two amino acids in any order, although longer peptides that contain lysine and arginine may also be used. Lysine and arginine containing peptides used as amino terminal capping groups in the peptide compounds described herein may be conveniently incorporated into whatever process is used to synthesize the peptide compounds to yield the derived peptide compound containing the amino terminal capping group.

[0059] In another embodiment of the invention, the peptides may contain a carboxy terminal capping group. The primary purpose of this group is to prevent intramolecular cyclization or inactivating intermolecular crosslinking or polymerization. Furthermore, a carboxy terminal capping group may provide additional benefits to the peptide, such as enhanced efficacy, reduced side effects, enhanced antioxidative activity, and/or other desirable biochemical properties. An example of such a useful carboxy terminal capping group is a primary or secondary amine in an amide linkage to the carboxy terminal amino acid residue. Such amines
may be added to the Q-carboxyl group of the carboxy terminal amino acid of the peptide using standard amidation chemistry.

[0060] In a further embodiment of the invention, these peptide compounds may contain one or more D-amino acid residues in place of one or more L-amino acid residues provided that the incorporation of the one or more D-amino acids does not abolish all or so much of the activity of the peptide compound that it cannot be used in the compositions and methods of the invention. Incorporating D-amino acids in place of L-amino acids is favorable as it may provide additional stability to a peptide compound.

[0061] In another embodiment of the present invention, conservative amino acid substitution in the sequence of the peptides may be performed. Amino acid substitution may be performed insofar as the exchange of amino acid residues occurs from within one of the following groups of residues: Group 1, representing the small aliphatic side chains and hydroxyl group including Ala, Gly, Ser, Thr, and Pro; Group 2, representing OH and SH side chains including Cys, Ser, Thr and Tyr; Group 3, representing residues which have carboxyl containing side chains such as Glu, Asp, Asn and Gm; Group 4, representing basic side chains including His, Arg and Lys; Group 5, representing hydrophobic side chains including He, Val, Leu, Phe and Met; and Group 6, representing aromatic side chains including Phe, Trp, Tyr and His.

[0062] The invention also provides for a peptide in a monovalent state. The composition may be as a free peptide or a single peptide fragment coupled to a carrier molecule. The peptides may also be used as conjugates having more than one peptide fragment bound to a single carrier molecule. The carrier may be a biological carrier such as a glycosaminoglycan, a proteoglycan, or albumin, or it may be a synthetic polymer such as a polyalkyleneglycol or a synthetic chromatography support. Other carriers include ovalbumin and human serum albumin, other proteins, and polyethylene glycol.

[0063] Still other carriers that may be used in the pharmaceutical compositions of this invention include ion exchangers, alumina, aluminum stearate, lecithin, non-albumin serum proteins, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyyxypolyylene-block polymers, and wool fat. Such modifications may both increase the apparent affinity and change the stability of a peptide. Although the number of peptide
fragments bound to each carrier can vary, typically about 4 to 8 peptide fragments per carrier molecule are bound under standard coupling conditions.

[0064] Alternatively, peptidomimetic compounds, may be designed based upon the amino acid sequences of the peptides of the current invention. The peptidomimetic compounds herein are synthetic compounds with three-dimensional conformation substantially similar to the three-dimensional conformation of the selected peptides of the invention. The peptide motif provides the peptidomimetic compound with the ability to suppress an immune response in a manner qualitatively identical to that of the peptide fragment from which the peptidomimetic was derived. Furthermore, the peptidomimetic compounds might have additional characteristics that enhance their therapeutic utility, such as increased cell permeability and a prolonged biological half-life.

[0065] The backbone of the peptidomimetics are partially or completely non-peptide, but their side groups are identical to the side groups of the amino acid residues that occur in the peptide on which the peptidomimetics are based. Several types of chemical bonds, e.g., ester, thioester, thioamide, retroamide, reduced carbonyl, dimethylene and ketomethylene bonds, are known in the art to be generally useful substitutes for peptide bonds in the construction of protease-resistant peptidomimetics.

[0066] The peptide can also be dimerized wherein the dimer comprises a disulfide bond. The peptide can be any of the following: pegilated, heterologous, labeled with a detectable marker, linked to a solid phase substrate or conjugated at a free amine group with a polyalkylene glycol.

[0067] Alternatively, the peptides in the following invention can be prepared using recombinant DNA technology in which a DNA sequence coding for the polypeptide is linked to an expression vector and used to transform an appropriate host cell. The transformed host cell is cultured and the polypeptide is expressed. The polypeptide is then recovered from the culture. Further still, a combination of synthesis and recombinant DNA techniques can be employed to produce the amide and ester derivatives of this invention, as well as to produce fragments of the desired polypeptide which are then assembled by methods well known to those skilled in the art.

[0068] In another embodiment, the peptides in the current invention can be synthesized using standard methods known in the art. Direct synthesis of the peptides of the invention may be accomplished using solid-phase peptide synthesis, solution-phase synthesis or other conventional means. For example, in solid-phase synthesis, a suitably protected amino acid residue is attached through its carboxyl group to an insoluble polymeric support, such as a cross-linked polystyrene or polyamide resin. In our context, a protected amino acid refers to the
presence of protecting groups on both the amino group of the amino acid, as well as on any side
chain functional groups. The benefit of side chain protecting groups are that they are generally
stable to the solvents, reagents, and reaction conditions used throughout the synthesis and are
removable without affecting the final peptide product. Typically, stepwise synthesis of the
polypeptide is carried out by the removal of the N-protecting group from the initial carboxy
terminal and coupling it to the next amino acid in the sequence of the polypeptide. The carboxyl
group of the incoming amino acid can be activated to react with the N-terminus of the bound
amino acid by formation into a reactive group such as formation into a carbodiimide, a
symmetric acid anhydride, or an active ester group such as hydroxybenzotriazole or
pentamorophenyl esters. The solid-phase peptide synthesis methods include both the BOC and
FMOC methods, which utilizes tert-butloxyxycarbonyl, and 9-fluorenlymethloxyxycarbonyl as the
α-amino protecting groups, respectively, both well-known by those of skill in the art (Sambrook
Spring Harbor, N.Y.; Ausubel et al., Current Protocols in Molecular Biology, John Wiley and
Sons, New York, 1995).

[0069] In another embodiment of the invention, the peptides may also be prepared and stored in
a salt form. Various salt forms of the peptides may also be formed or interchanged by any of the
various methods known in the art, e.g., by using various ion exchange chromatography methods.
Cationic counter ions that may be used in the compositions include, but are not limited to,
amines, such as ammonium ions, metal ions, especially monovalent, divalent, or trivalent ions of
alkali metals including sodium, potassium, lithium, cesium; alkaline earth metals including
calcium, magnesium, barium; transition metals such as iron, manganese, zinc, cadmium,
molybdenum; other metals like aluminum; and possible combinations of these. Anionic counter
ions that may be used in the compositions described below include chloride, fluoride, acetate,
trifluoroacetate, phosphate, sulfate, carbonate, citrate, ascorbate, sorbate, glutarate,
ketoglutarate, and possible combinations of these. Trifluoroacetate salts of peptide compounds
described here are typically formed during purification in trifluoroacetic acid buffers using high-
performance liquid chromatography (HPLC). Although usually not suited for in vivo use,
trifluoroacetate salt forms of the peptides described in this invention may be conveniently used
in various in vitro cell culture studies, assays or tests of activity or efficacy of a peptide
compound of interest. The peptide may then be converted from the trifluoroacetate salt by ion
exchange methods or synthesized as a salt form that is acceptable for pharmaceutical or dietary
supplement compositions.
[0070] Peptides according to the invention may also be prepared commercially by companies providing peptide synthesis as a service (e.g., BACHEM Bioscience, Inc., King of Prussia, Pa.; AnaSpec, Inc., San Jose, California). Automated peptide synthesis machines, such as manufactured by Perkin-Elmer Applied Biosystems, also are available.

[0071] The peptides useful in the methods of the present invention are purified once they have been isolated or synthesized by either chemical or recombinant techniques. Standard methods for purification purposes can be used, including reversed-phase high-pressure liquid chromatography (HPLC) using an alkylated silica column such as C4, C2- or C18-silica. In this method, a gradient mobile phase of increasing organic content is generally used to achieve purification, for example, acetonitrile in an aqueous buffer, usually containing a small amount of trifluoroacetic acid. Alternatively, ion-exchange chromatography can also be used to separate peptide compounds based on their charge. The degree of purity of the peptide compound may be determined by the identification of a major large peak on HPLC. A preferred level of purity results from a peptide that produces a single peak that is at least 95% of the input material on an HPLC column. More is a polypeptide that produces a single peak that is at least 97%, to 99.5% of the input material on an HPLC column.

**Vaccine Preparations**

[0072] The peptides from the present invention can be used to formulate vaccine compositions useful in preventing transmission of HIV-2. Such vaccine compositions contain an HIV peptide, fragment or analog of the invention, combinations thereof or multivalent peptide constructs of the invention. Such a vaccine composition may contain the HIV peptide or construct according to the invention together with a pharmaceutically acceptable carrier or diluent suitable for administration as a composition for prophylactic treatment of HIV infections. Suitable pharmaceutically acceptable carriers, as described above, facilitate administration of the proteins but are physiologically inert and/or nonharmful.

[0073] This vaccine composition may contain one, or preferably more than one, non-transmissible HIV peptide, fragment and/or peptide constructs of the invention which is associated with non-transmission status. Such a vaccine may be useful in uninfected individuals to induce protective titers of neutralizing anti-HIV antibodies. In one embodiment, a vaccine composition may contain a cocktail of both individual peptides rgp36 and rpC2-C3. In another embodiment multiple copies of a single HIV peptide of this invention, or multiple copies of more than one of these peptides may be employed in a single construct. For example, the peptides may be fused in frame to each other, or via a linker. Alternatively, multiple HIV
peptides of this invention may be present in a multivalent peptide construct which contains multiple copies of one or both of the peptides of this invention fused or linked to an inert carrier, to another protein, or to a lysine core.

[0074] An alternative, desirable vaccine composition may contain a conventional bio-expression vector, such as an adenovirus, poliovirus, vaccinia virus or retrovirus, into which the sequences of one or both of the HIV peptides, or functional fragments thereof are inserted under the control of the viral expression regulatory sequences [see, e.g., U.S. Pat. No. 4,920,209]. Such viral vector compositions can be employed to deliver effective doses of the vaccine as a therapeutic agent to an infected subject or as a prophylactic composition to a seronegative individual.

[0075] Optionally, the vaccine composition may further contain adjuvants, preservatives, chemical stabilizers, or other antigenic proteins. Typically, stabilizers, adjuvants, and preservatives are optimized to determine the best formulation for efficacy in the vaccinee. Suitable preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallade, the parabens, ethyl vanillli, glycerin, phenol, parachlorophenol.

[0076] One or more of the above described vaccine components may be admixed or adsorbed with a conventional adjuvant. The adjuvant is used as a non-specific irritant to attract leukocytes or enhance an immune response. Such adjuvants include, among others, mineral oil and water, BCG, aluminum hydroxide, Avridine, L121/squalene, D-lactide-polylactide/glycoside, muramyl dipeptide, killed Bordetella, saponins, as Quil A.

[0077] Suitable amounts of the active ingredient can be determined by one of skill in the art based upon the level of immune response desired. In general, however, the vaccine composition contains between 1 ng to 1000 mg peptide and more preferably, 0.05 mg to 1 mg per mL, whether a single HFV peptide of the invention or a combination of these peptides or peptide constructs is employed. Antigens to other pathogens, such as measles, mumps, and rubella (MMR) vaccines, may be combined in a vaccine composition of the invention. Suitable doses of the vaccine composition of the invention can be readily determined by one of skill in the art. Generally, a suitable dose is between 0.1 to 5 mL of the vaccine composition. Further, depending upon the subject being treated, i.e. weight, age, sex and general health, the dosage can also be determined readily by one of skill in the art.

[0078] The present invention also provides a prophylactic method entailing administering to a subject an effective amount of such a composition. For example, for prevention of vertical transmission, a vaccine composition of the invention could be administered either to a pregnant HIV-infected woman or an HIV-infected woman of child-bearing age. In general, the vaccine
will be administered once, or preferably, more frequently depending on the likelihood of exposure to the virus. Where desirable, boosters may be administered. The vaccine may be administered by any suitable route. However, parenteral administration, particularly intramuscular, and subcutaneous, is the preferred route. Also preferred is the oral route of administration.

[0079] The present invention further provides pharmaceutical compositions useful in providing passive immunity against HIV. Such compositions are useful for administration to subjects anticipating risk of exposure to the HIV virus, e.g., prior to surgery or for health-care workers.

[0080] The vaccines described herein may further contain other active ingredients including, for example, biological response modifiers, such as interleukins, colony stimulating factors, especially GM-CSF, IFNs, and other immunostimulatory cytokines, as well as preservatives, or chemical stabilizers.

[0081] Suitable dosages can be determined by the attending physician, with reference to the discussion herein relating to appropriate doses for the therapeutic and vaccinal compositions of the invention. The composition administration may be by any appropriate route and repeated as necessary.

**Antibodies**

[0082] The peptides from the present invention can also be used to generate antibodies capable of recognizing and binding naturally-occurring HIV epitopes when the virus or particles thereof are present in a biological fluid of a subject. These antibodies may be generated by conventional means utilizing the peptides of this invention (See, e.g., PCT Application WO91/04273). For example, polyclonal antibodies may be generated by conventionally stimulating the immune system of a selected animal with one or both of the above-identified peptides, or multivalent constructs, allowing the immune system to produce natural antibodies thereto, and collecting these antibodies from the animal's blood or other biological fluid. High titer polyclonal antibodies may be obtained by using the multivalent constructs described above as antigens. The resulting antibodies are capable of binding the selected HIV antigen as it appears in the biological fluids of an infected subject.

[0083] Additionally, the peptides of the present invention may also be used to generate antibodies that can be used as templates to generate anti-idiotype antibodies having the internal image of the neutralizing epitope structure contained in the peptide sequence. These antibodies, polyclonal or monoclonal, can then be used in vaccine formulations or in active immunotherapy. Accordingly, the present invention also includes monoclonal or polyclonal antibodies that carry
the internal image of the peptides, as well as methods for generating these antibodies [PCT/US90/05393]. This published application is incorporated by reference herein, with particular reference to the discussion of antibodies therein.

Where it is desirable to obtain and utilize monoclonal antibodies (MAb) for the compositions and the methods of this invention, hybridoma cell lines expressing desirable MAbs may be generated by well-known conventional techniques, e.g. Kohler and Milstein, and using available tumor cell lines.


These peptides, fragments and antibodies of this invention are thus useful as diagnostic reagents and vaccine components useful in the prophylaxis of HIV. The peptides and antibodies thereto may be associated with a diagnostic label, a chemical moiety, a toxin, another protein or peptide, provided that the peptide associated with such a molecule is characterized by substantially the same biological activity as the original peptide.

**Examples**

The following examples are set forth to assist in understanding the invention and should not, of course, be construed as specifically limiting the invention described and claimed herein. Such variations of the invention, including the substitution of all equivalents now known or later developed, which would be within the purview of those skilled in the art, and changes in formulation or minor changes in experimental design, are to be considered to fall within the scope of the invention incorporated herein.

**EXAMPLE 1: Clinical Specimens**

AU plasma samples used in this study were obtained from Portuguese hospitals. The samples were collected according to the country guidelines and relevant institutional policies. The plasma samples were stored at -80 °C. HIV seropositivity was determined by using the licensed kit VIDAS HIV DUO (Bio-Merieux). HIV-1 and HIV-2 differentiation was done by Western blot (Western blot) 2.2 (Genelabs Diagnostics), New LAV Blot II and Peptilav 1-2 (Bio-Rad). Western blot results were considered positive when two Env bands with or without
Gag and/or Pol bands, were present (19). Western blot results were considered negative when no HIV specific band was present and indeterminate when it showed any band pattern not considered positive or negative.

**EXAMPLE 2: Cloning and expression of rgp36 and rpC2-C3 polypeptides**

[0089] Using pSK7.3 plasmid as template, which contains HIV-2ALI env gene (55), a PCR was performed with primers Hepit 11 (5'-TTTAGATATGCAGTGACC-S') and Hepit 12 (5'-TTAGATCCACATATATAC-3') to obtain a C2-C3 env fragment with 497-pb (positions 661 to 1157 in HIV-2ALI env). Thermal cycling conditions were as follows: denaturation 94°C, 1 min; annealing 60°C, 1 min; extension 72°C, 1 min for 45 cycles. Another PCR was performed with primers Hepit 15 (5'-GGCACGGCAGCTTTAACGC-S') and Hepit 17 (5'-GTCCCGGAGTTATTTTGTAGTTCATATG-3') to obtain a gp36 fragment with 385-pb (positions 1578 to 1963 in HIV-2ALI env). Thermal cycling conditions were as follows: denaturation 94°C, 1 min; annealing 65°C, 1 min; extension 72°C, 1 min for 40 cycles. The resulting fragments were cloned into the bacterial expression vector pTrcHis (Invitrogen), generating recombinant plasmids pTrcC2-C3 and pTrcgp36. The expression of both recombinant polypeptides rpC2-C3 and rgp36 in Escherichia coli strain TOPIO was induced with isopropyl-β-D-thiogalactopyranoside following the instructions from the manufacturer. Briefly, 10 ml of Luria's broth supplemented with 0.1mg/ml ampicillin and 0.5% glycerol was inoculated with E. coli strain TOPIO harbouring plasmid pTrcC2-C3 or pTrcgp36, and incubated during 17 h at 37 °C with shaking (250 rpm). This culture was inoculated into 1000 ml of Luria's broth and incubated again at 37 °C with shaking until cells reached an optical density (600 nm) of 0.6. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 niM, and the expression was induced during 2 hours. The cells were harvested by centrifugation at 6000 rpm/10 minutes and the biomass was resuspended and homogenized in 40 ml of 6 M guanidine hydrochloride, 0.02 M sodium phosphate and 0.5 M NaCl, pH 7.8. The suspension was sonicated with three 10-second pulses at a high intensity setting to shear the DNA and RNA, and centrifuged at 10000 rpm/15 min. The insoluble debris were removed by low speed centrifugation and the supernatant was collected.

**EXAMPLE 3: Purification of recombinant polypeptides**

[0090] Purification of the histidinated rgp36 and rpC2-C3 polypeptides was done using a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia). A total of 40 ml clear supernatant was loaded at a flow rate of 1 ml/min onto ProBond™ resin (Invitrogen) column (1.6 x 20 cm, 10 ml bed volume) previously packed and equilibrated in 8 M urea, 0.02 M sodium phosphate
and 0.5 M NaCl, pH 7.8 (buffer A). After washing the column with buffer A to eliminate the unspecific proteins, the absorbed proteins were eluted with a pH-step gradient (pH 7.8 - 4) at a flow-rate of 1 ml/min according to the manufacturer's instructions. The purified recombinant polypeptides were analysed by sodium dodecyl sulphate- 12% polyacrylamide gel electrophoresis under reducing conditions to determine the size of the fusion proteins. Quantification of the purified proteins was done with the Bio-Rad Protein Assay (Bio-Rad).

**EXAMPLE 4: ELISA-HIV2 assay**

[0091] Polystyrene immune modules microwells (Maxisorp; Nalgen Nunc International), were independently coated (100 µl/well) with each recombinant polypeptide at a concentration of 2.5 µg/ml in 0.05 M bicarbonate buffer, pH 9.4 and incubated overnight at 4°C. After one wash with 0.01 M Tris and 0.15 M NaCl, pH 7.4 (TBS), micro wells were blocked with 1% gelatine (Bio-Rad) during 1 h. After two washes with TBS buffer, 100 µl of 1/100 dilution of each HIV positive and negative plasma samples in TBS containing 0.05 % Tween-20 (TBS-T), 0.1% gelatine and 5% goat serum (Sigma-Aldrich) was added and incubated 1 h at room temperature (RT). After washing five times with TBS-T, a 1:2000 dilution of goat anti-human immunoglobulin G (Fc specific) conjugated to alkaline phosphatase (Sigma-Aldrich) in TBS-T was added and incubated for 1 h at RT. The color was developed using p-nitrophenilphosphate (p-NPP Tablets, Sigma-Aldrich) as chromogenic substrate and the optical density (OD) was measured with an automated microplate reader LP 400 (Bio-Rad) at 405 nm against a reference wavelength of 620 nm. The clinical cut-off value of the assay, calculated as the mean OD value of HIV-seronegative samples plus three times the standard deviation [SD], was determined using samples from healthy HIV-seronegative subjects (n = 60). The results of the assay are expressed quantitatively as OD_{clinical sample} / OD_{cut-off} ratios. For ratio values > 1 the sample is considered as seroreactive.

**EXAMPLE 5: PCR amplification and viral load**

[0092] Proviral DNA was extracted from uncultured peripheral blood mononuclear cells with the Wizard® Genomic DNA Purification kit (Promega). For HIV-1, nested PCR was used to amplify a 409-bp fragment from the C2-C3 env region, using outer primer pairs JA1 67 and JA170 and inner primers JA168 and JA169, and a 582-bp fragment from the pl7 gag region, using outer primer pairs JA1 52 and JA1 55 and inner primers JA1 53 and JA1 54. Thermal cycling conditions for PCR and primer numbers and positions have been described previously (32). For HIV-2, nested PCR was used to amplify a 378-bp fragment from the HIV-2 C2-C3 env gene region (positions 6949 to 7327 in HIV-2ALI) as described elsewhere (4). The
amplified PCR products were visualized by electrophoresis in 2% agarose gel. For each subject, at least two independent PCR reactions were performed under identical conditions. HIV-I plasma viral load was determined using the Quantiplex HIV RNA 3.0 (bDNA) kit (Bayer Diagnostics).

**EXAMPLE 6: Statistical analyses**

[0093] Statistical analyses were performed with GraphPad Prism software (version 4.03; GraphPad Software, Inc., San Diego). The Student t test was used to compare mean S/CO OD values obtained with HIV-I and HIV-2 samples for both antigens (rgp36 and rpC2-C3). The Spearman rank test was used to analyse the association between the antibody responses to rgp36 and rpC2-C3 in HIV-2 infected subjects.

**EXAMPLE 7: Immunogenicity of rgp36 and rpC2-C3**

[0094] The recombinant histidinated polypeptides rgp36 and rpC2-C3 were expressed in *E. coli* and purified to 95% homogeneity. Final concentrations of 7 and 3.4 mg/l were obtained for rgp36 and rpC2-C3, respectively. The immunoreactivity of rgp36 and rpC2-C3 was determined by Western blot analysis with a panel of HIV-2 or HIV-I seropositive samples and nine seronegative samples from Portugal. All HIV-2 samples reacted with rgp36 and rpC2-C3, whereas none of the HIV-I or seronegative samples did. These results demonstrated that both envelope regions are highly immunogenic in HIV-2 infected individuals, and suggested that the recombinant polypeptides could be useful antigens both for serological diagnosis of HIV-2 infection and to discriminate between HIV-I and HIV-2 infections.

**EXAMPLE 8: Development of HIV-2 Specific ELISA**

[0095] A microplate ELISA assay, ELISA-HIV2, was developed using rgp36 and rpC2-C3 polypeptides as independent capture antigens. The clinical specificity of ELISA-HIV2 was evaluated against a panel of plasma samples from healthy HIV seronegative subjects. These included samples from blood donors (n = 130) and from pregnant women (n = 30). Two samples reacted weakly against rgp36 (mean S/CO ratio, 1.21; SD, 0.27); 8 samples reacted weakly against rpC2-C3 (mean S/CO ratio, 1.18; SD, 0.23). Upon retesting in duplicate, all samples gave negative results. Therefore, 100% clinical specificity was obtained for both polypeptides. A panel of 106 HIV-2 positive samples was used to determine the clinical sensitivity of the ELISA-HIV2 assay. All 106 samples reacted with rgp36 and 99 (93.4%) samples reacted also with rpC2-C3 (Fig. IA and Fig. IB). The 100% clinical sensitivity and specificity obtained with rgp36 indicates that the ELISA-HIV-2 assay can be used in the serological detection of HIV-2 infection.
Mean S/CO ratio was significantly higher for the rgp36 antigen compared to rpC2-C3 (8.27; SD, 1.49 vs. 4.89; SD, 2.51; PO.0001) (Fig. 1C). HIV-2 subjects could be clustered into high (HR) and low (LR) immune responders according to the level of antibodies to rpC2-C3 (Fig. 1D). The Spearman Rank test and linear regression were used to analyse the correlation between the antibody responses of HIV-2 infected subjects (n = 99) to the rpC2-C3 and rgp36 polypeptides. There was a significant positive correlation between the immune response to both peptides \( r^2 = 0.259, \) Spearman \( r = 0.4740, \) P<0.0001) (Fig. 2). Taken together, these results show that the antibody response to the HIV-2 envelope in infected subjects is conceitedly directed to the gp36 ectodomain and the gpl25 C2-C3 region, and suggest that the gp36 ectodomain is the immunodominant antigenic region in the HIV-2 envelope. Moreover, our results suggest that the level of anti-C2-C3 antibodies may be a correlate of immune function in HIV-2 infection. All 95 HIV-I samples analysed with ELISA-HIV2 gave negative results with rpC2-C3. 31 (32.6%) samples cross-reacted with rgp36 but the reactivity was significantly weaker when compared with that of HIV-2 samples (mean S/CO ratio, 2.42; SD, 0.85 vs. 8.27, SD, 1.49; P<0.0001) (Fig. 1A). These results suggested that the ELISA-HIV2 assay could be useful to discriminate between HIV-I and HIV-2 infection in individuals with dual positive serology. Seven HIV-I and HIV-2 dually reactive serum samples were analysed by ELISA-HIV2, and PCR amplification of HIV gag and/or env genes (Table 2).
TABLE 2 - Type of infection in dually HIV-1 and HIV-2 seroreactive individuals determined with ELISA-HIV2 and PCR amplification

<table>
<thead>
<tr>
<th>Sample</th>
<th>ELISA-HIV2 (S/CO)</th>
<th>Viral load</th>
<th>PCR amplification</th>
<th>Type of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HTV-1</td>
<td>HIV-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gag</td>
<td>env</td>
</tr>
<tr>
<td>107</td>
<td>9.35</td>
<td>11.87</td>
<td>&lt;50</td>
<td>-</td>
</tr>
<tr>
<td>108</td>
<td>8.53</td>
<td>1.89</td>
<td>&lt;50</td>
<td>-</td>
</tr>
<tr>
<td>109</td>
<td>8.68</td>
<td>4.11</td>
<td>&lt;50</td>
<td>-</td>
</tr>
<tr>
<td>110</td>
<td>2.58</td>
<td>0.55</td>
<td>241</td>
<td>-</td>
</tr>
<tr>
<td>111</td>
<td>8.25</td>
<td>3.32</td>
<td>&lt;50</td>
<td>-</td>
</tr>
<tr>
<td>112</td>
<td>0.51</td>
<td>0.49</td>
<td>47273</td>
<td>+</td>
</tr>
<tr>
<td>113</td>
<td>0.80</td>
<td>0.50</td>
<td>35123</td>
<td>+</td>
</tr>
</tbody>
</table>

1 RNA copies/ml plasma; 2 Amplification of p17 (gag) and C2-C3 (env) regions; (-), negative; (+), positive.

[0097] The PCR amplification results were 100% concordant with the ELISA-HIV2 results in four subjects which were, therefore, considered as HIV-2 infected. The remaining three subjects were HIV-1 infected judging by the negative ELISA-HIV2 and HIV-2 PCR results, and positive HIV-1 PCR reactions and plasma viral load. Therefore, the ELISA-HIV2 assay can be used to discriminate between HIV-1 and HIV-2 infections in dually seroreactive subjects.

[0098] Western blot confirmatory results are considered positive when 2 Env bands are present (19). To investigate the reliability of ELISA-HIV2 as a confirmatory test, tested a panel of samples (n = 56) that were reactive in the screening assay VIDAS HIV DUO and in the confirmatory New LAV Blot II (51 positive and 5 indeterminate) were tested. All 51 Western blot positive samples reacted as HIV-2 samples in ELISA-HIV2 whereas the Western blot indeterminate samples were HIV-2 negative in ELISA-HIV2 (Table 3).
TABLE 3 - Diagnostic performance of ELISA-HIV-2 with samples classified as indeterminate in Western blot

<table>
<thead>
<tr>
<th>Sample</th>
<th>ELISA-HIV2 (S/CO)</th>
<th>New LAV Blot II</th>
<th>Peptilav 1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rgp36</td>
<td>rpC2-C3</td>
<td>Band pattern on HTV-2 blot</td>
</tr>
<tr>
<td>53</td>
<td>0.77</td>
<td>0.45</td>
<td>p26</td>
</tr>
<tr>
<td>62</td>
<td>0.63</td>
<td>0.55</td>
<td>p68, gp36, p26</td>
</tr>
<tr>
<td>68</td>
<td>1.70</td>
<td>0.42</td>
<td>gp36, p26</td>
</tr>
<tr>
<td>76</td>
<td>2.57</td>
<td>0.58</td>
<td>(gp140, gp105, p68, p26)*</td>
</tr>
<tr>
<td>92</td>
<td>3.43</td>
<td>0.50</td>
<td>p68, p26</td>
</tr>
</tbody>
</table>

* Faint bands; (+), positive; (-), negative; S/CO - ODsample / ODcut-off

[0099] With the Peptilav 1-2 assay all indeterminate samples reacted with the HIV-I gp41 peptide indicating that they were HIV-I positive. These results demonstrate that the ELISA-HIV2 assay can be used as a confirmatory assay for the serological diagnosis of HIV-2 infection.
CLAIMS

1. A recombinant gp36 protein comprising at least one ectopic polypeptide linked to a gp36 polypeptide or peptide fragment thereof, wherein the gp36 polypeptide or peptide fragment thereof comprises at least 30 amino acids from the amino acid sequence of SEQ ID NO: 1.

2. The recombinant gp36 protein of claim 1, wherein the ectopic polypeptide is selected from the group consisting of glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides.

3. The recombinant gp36 protein of claim 2, wherein the ectopic polypeptide is at least one poly-histidine tag.

4. The recombinant gp36 protein of claim 1, wherein the gp36 polypeptide comprises at least 35 amino acids of SEQ ID NO:1.

5. The recombinant gp36 protein of claim 1, wherein the gp36 polypeptide comprises at least 40 amino acids of SEQ ID NO:1.

6. The recombinant gp36 protein of claim 1, wherein the gp36 polypeptide comprises at least 45 amino acids of SEQ ID NO:1.

7. The recombinant gp36 protein of claim 1, wherein the gp36 polypeptide comprises at least 50 amino acids of SEQ ID NO:1.

8. The recombinant gp36 protein of claim 1, wherein the gp36 polypeptide comprises at least 55 amino acids of SEQ ID NO:1.

9. The recombinant gp36 protein of claim 1, wherein the gp36 polypeptide is at least 95% homologous to the amino acid sequence from SEQ ID NO:1.

10. The recombinant gp36 protein of claim 1 furthering comprising a peptide linker sequence linking the gp36 polypeptide or fragments thereof to at least one ectopic polypeptide.

11. A polypeptide comprising at least 30 amino acids from the amino acid sequence of the C2-C3 envelope protein of HIV-2 represented as SEQ ID NO:2.

12. The polypeptide of claim 11, wherein the polypeptide is at least 35 amino acids of SEQ ID NO:2.

13. The polypeptide of claim 11, wherein the polypeptide is at least 40 amino acids of SEQ ID NO:2.
14. The polypeptide of claim 11, wherein the polypeptide is at least 45 amino acids of SEQ ID NO:2.
15. The polypeptide of claim 11, wherein the polypeptide is at least 50 amino acids of SEQ ID NO:2.
16. The polypeptide of claim 11, wherein the polypeptide is at least 95% homologous to the amino acid sequence of SEQ ID NO:2.
17. The polypeptide of claims 11-16, wherein the polypeptide further comprises at least one ectopic polypeptide linked to the C2-C3 polypeptide so as to form a recombinant C2-C3 protein.
18. The recombinant C2-C3 protein of claim 17, wherein the ectopic polypeptide is selected from the group consisting of glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides.
19. The recombinant C2-C3 protein of claim 18, wherein the ectopic polypeptide is at least one poly-histidine tag.
20. The recombinant C2-C3 protein of claim 17, wherein the C2-C3 polypeptide comprises at least 35 amino acids of SEQ ID NO:2.
21. The recombinant C2-C3 protein of claim 17, wherein the C2-C3 polypeptide comprises at least 40 amino acids of SEQ ID NO:2.
22. The recombinant C2-C3 protein of claim 17, wherein the C2-C3 polypeptide comprises at least 45 amino acids of SEQ ID NO:2.
23. The recombinant C2-C3 protein of claim 17, wherein the C2-C3 polypeptide comprises at least 50 amino acids of SEQ ID NO:2.
24. The recombinant C2-C3 protein of claim 17, wherein the C2-C3 polypeptide comprises at least 55 amino acids of SEQ ID NO:2.
25. The recombinant C2-C3 protein of claim 17, wherein the C2-C3 polypeptide is at least 95% homologous to the amino acid sequence from SEQ ID NO:2.
26. The recombinant C2-C3 protein of claim 17 furthering comprising a peptide linker sequence linking the C2-C3 polypeptide or fragments thereof to at least one ectopic polypeptide.
27. A biological assay comprising a recombinant gp36 protein and a recombinant C2-C3 protein immobilized on a solid substrate.
28. The biological assay of claim 27, wherein the recombinant gp36 protein comprises at least one ectopic polypeptide linked to a gp36 polypeptide or fragment thereof from SEQ ID NO:1.

29. The biological assay of claim 28, wherein the ectopic polypeptide is selected from the group consisting of glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides.

30. The biological assay of claim 29, wherein the ectopic polypeptide comprises a poly-histidine tag.

31. The biological assay of claim 27, wherein the recombinant C2-C3 protein comprises at least one ectopic polypeptide linked to a C2-C3 polypeptide or fragment thereof from SEQ ID NO:2.

32. The biological assay of claim 31, wherein the ectopic polypeptide is selected from the group consisting of glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides.

33. The biological assay of claim 32, wherein the ectopic polypeptide comprises a poly-histidine tag.

34. The biological assay of claim 27, wherein the recombinant gp36 protein is a poly-histidine-tagged gp36 polypeptide, the gp36 polypeptide being the amino acid sequence of SEQ ID NO:1, and the recombinant C2-C3 protein is a poly-histidine-tagged C2-C3 polypeptide, the C2-C3 polypeptide being an amino acid sequence of SEQ ID NO:2.

35. The biological assays of claims 27-24, wherein the solid substrate is selected from the group consisting of glass, polystyrene, PVDF membrane, nylon, and nitrocellulose.

36. A method of detecting or diagnosing HIV-2 in a subject, comprising:
(a) contacting a recombinant gp36 protein and a recombinant C2-C3 protein with a fluid sample isolated from a subject;
(b) allowing the anti-gp36 antibodies and anti-C2-C3 antibodies that are present in the fluid sample to specifically bind with the recombinant gp36 protein and the recombinant C2-C3 protein, and
(c) detecting the presence of anti-gp36 antibodies and anti-C2-C3 antibodies in the fluid sample,

wherein HIV-2 is indicated if the presence of anti-gp36 antibodies and anti-C2-C3 antibodies is detected in the fluid sample.
37. The method of claim 36, wherein the presence of anti-gp36 antibodies and anti-C2-C3 antibodies are detected by secondary antibodies that specifically bind to human IgG or human IgA.

38. The method of claim 37, wherein the secondary antibodies are labeled with a label selected from the group consisting of radiolabels, chemiluminescent labels, and fluorescent labels.

39. The method of claim 36, wherein the recombinant gp36 protein and the recombinant C2-C3 protein are immobilized on a solid substrate.

40. The method of claim 39, wherein the solid substrate is selected from the group consisting of glass, polystyrene, PVDF membrane, nylon, and nitrocellulose.

41. The method of claim 36, wherein the presence of anti-gp36 antibodies and anti-C2-C3 antibodies is detected by an enzyme-linked immunosorbent assay.

42. The method of claim 36, wherein the presence of anti-gp36 antibodies and anti-C2-C3 antibodies is detected by immunoblot, dot blot, or protein microarray.

43. The method of claim 37, further comprising:
(a) comparing the level of anti-C2-C3 antibodies detected in the fluid sample to a level of anti-C2-C3 antibodies detected in a control sample that is uninfected with HIV-2, wherein the presence of HIV-2 is indicated if the level of anti-C2-C3 antibodies detected in the fluid sample is greater than the level of anti-C2-C3 antibodies detected in the control sample.

44. A kit for diagnosing or detecting HIV-2 in a subject comprising: a) a recombinant gp36 protein; b) a recombinant C2-C3 protein; and (c) a detection means to detect anti-gp36 and anti-C2-C3 antibodies.

45. The kit of claim 44, wherein the recombinant gp36 protein comprises (a) an ectopic polypeptide; and (b) a gp36 polypeptide or fragment thereof of SEQ ID NO:1.

46. The kit of claim 45, wherein the ectopic polypeptide is selected from the group consisting of glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides.

47. The kit of claim 46, wherein the ectopic protein comprises a poly-histidine tag.

48. The kit of claim 44, wherein the recombinant C2-C3 protein comprises at least one ectopic polypeptide linked to a C2-C3 polypeptide or fragment thereof from SEQ ID NO:2.
49. The kit of claim 48, wherein the ectopic polypeptide is selected from the group consisting of glutathione sulfur transferase, poly-histidine tags, maltose-binding protein, HA-tags, c-Myc tags, and FLAG peptides.

50. The kit of claim 49, wherein the ectopic polypeptide comprises a poly-histidine tag.

51. The kit of claim 44, wherein the recombinant gp36 protein is a poly-histidine-tagged gp36 polypeptide, the gp36 polypeptide being the amino acid sequence of SEQ ID NO:1, and the recombinant C2-C3 protein is a poly-histidine-tagged C2-C3 polypeptide, the C2-C3 polypeptide being an amino acid sequence of SEQ ID NO:2.

52. The kit of claim 51, wherein the recombinant gp36 protein and the recombinant C2-C3 protein are immobilized on a solid support.

53. The kit of claims 44-52, wherein the solid substrate is selected from the group consisting of glass, polystyrene, PVDF membrane, nylon, and nitrocellulose.

54. The kit of claim 44, wherein the detection means are labeled secondary antibodies that specifically bind to IgG and/or IgA.

55. A vaccine for treating or preventing a HIV-2 infection, comprising (a) a gp36 polypeptide, or polypeptide subsequence thereof, and/or (b) a C2-C3 polypeptide, or polypeptide subsequence thereof, and (c) at least one pharmaceutically acceptable vaccine component.

56. The vaccine of claim 55, wherein the gp36 polypeptide or polypeptide subsequence is a polypeptide sequence of SEQ ID NO:1.

57. The vaccine of claim 56, wherein the gp36 polypeptide subsequence is at least ten amino acids long.

58. The vaccine of claim 56, wherein the gp36 polypeptide subsequence comprises a hapten.

59. The vaccine of claim 55, wherein the C2-C3 polypeptide or polypeptide subsequence is a polypeptide sequence of SEQ ID NO:2.

60. The vaccine of claim 59, wherein the C2-C3 polypeptide subsequence is at least ten amino acids long.

61. The vaccine of claim 59, wherein the C2-C3 polypeptide subsequence comprises a hapten.

62. The vaccine of claim 55, wherein the pharmaceutically acceptable vaccine component is an adjuvant.

63. A biological assay comprising:
(a) a recombinant gp36 protein consisting of a gp36 polypeptide linked to a poly-
histidine-tag, the gp36 polypeptide being the amino acid sequence of SEQ ID NO:1; and
(b) a recombinant C2-C3 protein linked to a poly-histidine-tag, the C2-C3 polypeptide
being an amino acid sequence of SEQ ID NO:2,
wherein the recombinant gp36 protein and the recombinant C2-C3 protein are
immobilized on a solid support.

64. A method of detecting or diagnosing HIV-2 in a subject, comprising:
(a) contacting a recombinant gp36 protein and a recombinant C2-C3 protein with a fluid
sample isolated from a subject;
(b) allowing the anti-gp36 antibodies and anti-C2-C3 antibodies that are present in the
fluid sample to specifically bind with the recombinant gp36 protein and the recombinant
C2-C3 protein;
(c) detecting the presence of anti-gp36 antibodies and anti-C2-C3 antibodies in the fluid
sample; and
(d) comparing the level of anti-C2-C3 antibodies detected in the fluid sample to a level
of anti-C2-C3 antibodies detected in a control sample that is uninfected with HIV-2,
wherein HIV-2 is indicated if the presence of anti-gp36 antibodies is detected in the fluid
sample and the level of anti-C2-C3 antibodies detected in the fluid sample is greater than
the level of anti-C2-C3 antibodies detected in the control sample.
**FIGURA 1A**

**rgp36**

$P < 0.0001$

**FIGURA 1B**

**rpC2-C3**

$P < 0.0001$
**Soros VIH-2**

P<0.0001

Polipéptidos recombinantes

**FIGURE 1C**

**RP C2-C3 reactividad**

P<0.0001

**FIGURA 1D**
$r^2 = 0.259$
Spearman $r = 0.474$
$P < 0.0001$

FIGURA 2