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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07H 21/04, A61K 48/00, 39/395, C12N 1/20 — A2

(11) International Publication Number: **WO 00/08043**

(43) International Publication Date: 17 February 2000 (17.02.00)

(21) International Application Number: PCT/US99/17487

(22) International Filing Date: 3 August 1999 (03.08.99)

(30) Priority Data:

60/095,105	3 August 1998 (03.08.98)	US
60/141,806	29 June 1999 (29.06.99)	US

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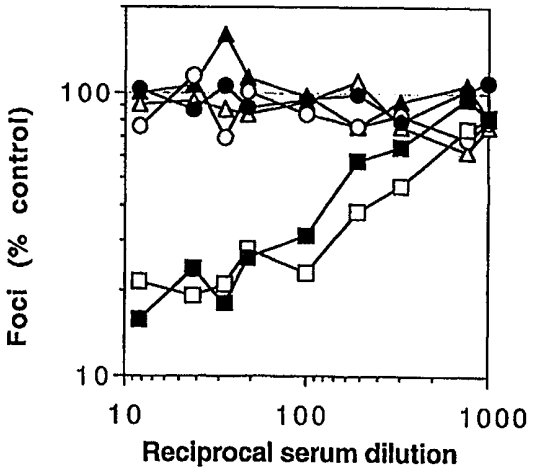
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: PREVENTION AND TREATMENT OF VIRAL DISEASE



(57) Abstract

The invention relates to the generation of Fusion–Related Molecular Structures (FRMS) which comprise one or more transitional fusion–related determinants. Highly effective vaccines may be constructed which present the fusion–related determinants to the immune system that engender unique antibodies capable of potently neutralizing a broad range of primary isolates, from worldwide locations and from different phylogenetic viral clades. The present invention provides methods for formation, isolation and purification of the FRMS as well as the use of such FRMS in a variety of compositions and methods, including, for example, as vaccine immunogens, diagnostics, and therapeutics. The present invention concerns FRMS capable of eliciting neutralizing antibodies to viral pathogens and primary isolates of a virus. Antibodies raised to the FRMS can be used to study the molecular pathway toward fusion of the virus and host cell and to identify points of a possible antibody–mediated blockade to that pathway. The invention also relates to the use of antibodies of the invention for anti–viral agents, blood product additives, contraceptive additives passive immunization in post–exposure treatments or fetus immunization.

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PREVENTION AND TREATMENT OF VIRAL DISEASE

The inventions consisting of monoclonal antibodies to the FRMS and affinity tagged viral envelope protein, respectively, were made with government support under grant numbers R01 AI44669-01 and R21 AI44312-02 awarded by the National Institutes of Health. The Government has certain rights in these inventions.

This application claims priority to U.S. Provisional Patent Application No. 60/095,105 filed August 3, 1998, and to U.S. Provisional Patent Application No. 60/141,806 filed June 29, 1999, both of which are incorporated herein by reference in their entireties.

1. INTRODUCTION

The present invention relates to the surprising discovery that highly effective vaccines may be constructed which present transitional fusion-related determinants to the immune system that engender unique antibodies capable of potently neutralizing a broad range of primary isolates, from worldwide locations and from different phylogenetic viral clades. The invention relates to the generation of Fusion-Related Molecular Structures (FRMS) which comprise one or more fusion-related determinants. The invention further relates to the discovery that the broad and uniform neutralization of diverse primary isolates indicates that the critical determinants presented by FRMS is highly conserved and may be intimately tied to the basic functioning of the envelope protein in binding and fusion. The present invention provides methods for formation, isolation and purification of the FRMS as well as the use of such FRMS in a variety of compositions and methods, including, for example, as vaccine immunogens, diagnostics, and therapeutics.

The present invention concerns FRMS capable of eliciting neutralizing antibodies to viral pathogens and primary isolates of a virus. Antibodies raised to the FRMS can be used to study the molecular pathway toward fusion of the virus and host cell and to identify points of a possible antibody-mediated blockade to that pathway. The invention also relates to the use of antibodies of the invention for anti-viral agents, blood product additives, contraceptive additives passive immunization in post-exposure treatments or fetus immunization.

2. BACKGROUND OF THE INVENTION

Citation of a reference herein shall not be construed as an admission that such reference is prior art to the present invention.

Viruses are infectious agents responsible for many diseases in humans, animals, bacteria and plants, and are therefore of great medical, and commercial importance. Broadly speaking, a free virus particle, called a virion, is made up of a genome (which can be RNA or DNA), associated proteins or polyamides, and a protein coat called a capsid. Some virions are further surrounded by a membranous envelope. Capsid and envelope structures serve to protect the genome from nucleases present in the environment and also serve to facilitate viral attachment and entry into the cell in which it will infect and replicate.

2.1. VIRAL ENTRY

A virus must utilize the biosynthetic machinery of the host cell to replicate. Viruses use the synthetic pathways and substrates available in animal cells to maintain and propagate their own genetic information. To gain access to the cell's biomachinery, the virus must enter or infect the cell. Entrance is facilitated by the presence of one or more viral receptor proteins on host cell membranes. Viral proteins bind to these receptors initiating a complex series of events by which the viral nucleic acid or nucleoproteins are released into the cell.

In the case of enveloped viruses, infection is facilitated by fusion of the viral envelope with a cellular membrane such as a cell surface membrane or an internal membrane. A variety of enveloped viruses are implicated in human and animal disease.

2.2. HIV

One clinically important example of an enveloped virus is HIV. The human immunodeficiency virus (HIV) has been implicated as the primary cause of the slowly degenerative immune system disease termed acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi, F., et al., 1983, Science 220:868-870; Gallo, R., et al., 1984, Science 224:500-503). In humans, HIV replication occurs prominently in $CD4^+$ T lymphocyte populations, and HIV infection leads to depletion of this cell type and eventually to immune incompetence, opportunistic infections, neurological dysfunctions, neoplastic growth, and ultimately death. At least two distinct types of HIV exist including: HIV-1 (Barre-Sinoussi, F., et al., 1983, Science 220:868-870; Gallo, R., et al., 1984, Science 224:500-503) and HIV-2 (Clavel, F., et al., 1986, Science 233:343-346; Guyader, M., et al., 1987, Nature 326:662-669). Additionally, significant genetic heterogeneity exists within populations of each types.

HIV is a member of the class of lentivirus in the family of retroviruses (Teich, N., et al., 1984, RNA Tumor Viruses, Weiss, R., et al., eds., CSH-Press, pp. 949-956). Retroviruses are small enveloped viruses that contain a single-stranded RNA genome,

and replicate via a DNA intermediate produced by a virally-encoded reverse transcriptase, an RNA-dependent DNA polymerase (Varmus, H., 1988, Science 240:1427-1439).

The HIV viral particle comprises a viral core, composed in part of capsid proteins, together with the viral RNA genome and those enzymes required for early replicative events. Myristylated gag protein forms an outer shell around the viral core, which, in turn, is surrounded by a lipid membrane envelope derived from an infected cell membrane. The HIV envelope surface glycoproteins are synthesized as a single 160 kilodalton precursor protein which is cleaved by a cellular protease during viral budding into two glycoproteins, gp41 and gp120. gp41 is a transmembrane glycoprotein and gp120 is an extracellular glycoprotein which remains non-covalently associated with gp41, possibly in a trimeric or multimeric form (Hammarskjold, M., et al., 1989, Biochem. Biophys. Acta 989:269-280).

HIV is targeted to $CD4^+$ cells because a CD4 cellular membrane protein (CD4) acts as a cellular receptor for the HIV-1 virus (Dalgleish, A., et al., 1984, Nature 312:763-767; Klatzmann et al., 1984, Nature 312:767-768; Maddon et al., 1986, Cell 47:333-348). Viral entry into cells is dependent upon gp120 binding the cellular CD4 receptor molecules (McDougal, J.S., et al., 1986, Science 231:382-385; Maddon, P.J., et al., 1986, Cell 47:333-348), explaining HIV's tropism for $CD4^+$ cells.

Upon interaction of the viral envelope protein and cellular receptor, the envelope protein–CD4 complex undergoes conformational changes facilitating subsequent interaction with host cellular co-receptors to form a trimolecular complex. Further conformational changes in the trimolecular complex allow fusion of the apposed cell membrane and virus envelope releasing viral genetic material into the cell.

2.3. VIRAL TREATMENT STRATEGIES

2.3.1. DRUG THERAPY

Although considerable effort is being put into the design of effective Therapeutics, currently no curative anti-retroviral drugs against AIDS exist. Attempts to develop such drugs have focused on several stages of the HIV life cycle (Mitsuya, H., et al., 1991, FASEB J. 5:2369-2381). Many viral targets for intervention with HIV life cycle have been suggested, since the prevailing view is that interference with a host cell protein would have deleterious side effects. For example, virally encoded reverse transcriptase has been one focus of drug development. A number of reverse-transcriptase-targeted drugs, including 2′,3′-dideoxynucleoside analogs such as AZT, ddI, ddC, and d4T have been developed which have been shown to been active against HIV (Mitsuya, H., et al., 1991, Science 249:1533-1544).

The new treatment regimens for HIV-1 demonstrate that a combination of anti-HIV compounds, which target reverse transcriptase (RT), such as azidothymidine (AZT), lamivudine (3TC), dideoxyinosine (ddI), dideoxycytidine (ddC) used in combination with an HIV-1 protease inhibitor have a far greater effect (2 to 3 logs reduction) on viral load compared to AZT alone (about 1 log reduction). For example, improved results have recently been obtained with a combination of AZT, ddI, 3TC and ritonavir (Perelson, A.S., et al., 1996, Science 15:1582-1586). However, it is likely that long-term use of combinations of these chemicals will lead to toxicity, especially to the bone marrow. Long-term cytotoxic therapy may also lead to suppression of $CD8^+$ T cells, which are essential to the control of HIV, via killer cell activity (Blazevic, V., et al., 1995, AIDS Res. Hum. Retroviruses 11:1335-1342) and by the release of suppressive factors, notably the chemokines Rantes, MIP-1α and MIP-1β (Cocchi, F., et al., 1995, Science 270:1811-1815). Another major concern in long-term chemical anti-retroviral therapy is the development of HIV mutations with partial or complete resistance (Lange, J.M., 1995, AIDS Res. Hum. Retroviruses 10:S77-82). It is thought that such mutations may be an inevitable consequence of anti-viral therapy. The pattern of disappearance of wild-type virus and appearance of mutant virus due to treatment, combined with coincidental decline in $CD4^+$ T cell numbers strongly suggests that, at least with some compounds, the appearance of viral mutants is an underlying factor in the failure of AIDS therapy.

Additionally, the failure of conventional drugs may also be attributable to reservoirs of HIV in slowly growing cell populations which are latent and do not actively produce virus and therefore escape many of the conventional treatments.

Attempts are also being made to develop drugs which can inhibit viral entry into the cell. For these studies, the focus has thus far focused on CD4, the cell surface receptor for HIV. Recombinant soluble CD4, for example, has been shown to inhibit infection of $CD4^+$ T cells by some HIV-1 strains (Smith, D.H., et al., 1987, Science 238:1704-1707). Certain primary HIV-1 isolates, however, are relatively less sensitive to inhibition by recombinant CD4 (Daar, E., et al., 1990, Proc. Natl. Acad. Sci. USA 87:6574-6579). In addition, recombinant soluble CD4 clinical trials have produced inconclusive results (Schooley, R., et al., 1990, Ann. Int. Med. 112:247-253; Kahn, J.O., et al., 1990, Ann. Int. Med. 112:254-261; Yarchoan, R., et al., 1989, Proc. Vth Int. Conf. on AIDS, p. 564, MCP 137).

The late stages of HIV replication, which involve crucial virus-specific processing of certain viral encoded proteins, have also been suggested as possible anti-HIV drug targets. Late stage processing is dependent on the activity of a viral protease, and drugs are being developed which inhibit this protease (Erickson, J., 1990, Science 249:527-533).

Recently, chemokines produced by $CD8^+$ T cells have been implicated in suppression of HIV infection (Paul, W.E., 1994, Cell 82:177; Bolognesi, D.P., 1993, Semin. Immunol. 5:203). The chemokines RANTES, MIP-1α and MIP-1β, which are secreted by $CD8^+$ T cells, were shown to suppress HIV-1 p24 antigen production in cells infected with HIV-1 or HIV-2 isolates *in vitro* (Cocchi, F, et al., 1995, Science 270:1811-1815). Thus, these and other chemokines may prove useful in therapies for HIV infection. The clinical outcome, however, of all these and other candidate drugs is still in question.

2.3.2. VACCINES

Another important strategy in anti-viral treatment and prevention is the development of vaccines. Due to the potential pandemic nature of viral infection, such as HIV, there is a significant need for effective vaccines.

Generally, traditional methods for preparing vaccines include the use of inactivated or attenuated pathogens. A suitable inactivation of the pathogenic microorganism renders it harmless as a biological agent but does not destroy its immunogenicity. Injection of these "killed" particles into a host will then elicit an immune response capable of preventing a future infection with a live microorganism. However, a major concern in the use of killed vaccines (using inactivated pathogen) is failure to inactivate all the microorganism particles. Even when this is accomplished, since killed pathogens do not multiply in their host, or for other unknown reasons, the immunity achieved is often incomplete, short lived and requires multiple immunizations. Finally, the inactivation process may alter the microorganism's antigens, rendering them less effective as immunogens.

Attenuation refers to the production of strains of pathogenic microorganisms which have essentially lost their disease-producing ability. One way to accomplish this is to subject the microorganism to unusual growth conditions and/or frequent passage in cell culture. Mutants are then selected which have lost virulence but yet are capable of eliciting an immune response. Attenuated pathogens often make good immunogens as they actually replicate in the host cell and elicit long lasting immunity. However, several problems are encountered with the use of live vaccines, the most worrisome being insufficient attenuation and the risk of reversion to virulence.

An alternative to the above methods is the use of subunit vaccines. This involves immunization only with those components which contain the relevant immunological material.

In the case of HIV, for example, envelope proteins (gp160, gp120, gp41) have been shown to be the major antigens for anti-HIV antibodies present in AIDS patients (Barin et al., 1985, Science 228:1094-1096). Thus far, therefore, these proteins have been

the most common candidates to act as immunogens for anti-HIV vaccine development. Several groups have begun to use various portions of gp160, gp120, and/or gp41 as immunogenic targets for the host immune system. See for example, Ivanoff, L., et al., U.S. Pat. No. 5,141,867; Saith, G., et al., WO92/22,654; Shafferman, A., WO91/09,872; Formoso, C., et al., WO90/07,119.

Because the HIV envelope protein mediates the early binding and entry steps in infection, many vaccine strategies have focused on blocking the function of the viral envelope glycoprotein. In 1993, two recombinant forms of the surface gp120 subunit of the HIV envelope protein (rgp120) were advanced as candidate vaccines for a large scale efficacy study sponsored by the National Institutes of Health (NIH). In previous clinical studies, these rgp120 vaccines had been shown to be safe and to elicit antibodies capable of potently neutralizing related laboratory-adapted isolates of HIV (Belshe, R.B., et al., 1994, Journal of the American Medical Association 272:475; Kahn, J.O., et al., 1994, Journal of Infectious Diseases 170:1288). Progress was stalled, however, by findings that Primary Isolate ("PI") viruses were largely refractory to neutralization by rgp120 vaccine sera (Cohen, J., 1993, Science 262:980; Cohen, J., 1994, Science 264:1839).

2.4. THE NEED FOR NEUTRALIZATION OF PRIMARY ISOLATES

The expanding epidemic of HIV infection threatens to infect more than 40 million persons worldwide by the year 2000 (UNAIDS Report (www.unaids.org/unaids/report). The need for an effective HIV vaccine is urgent, but progress towards this goal has been blocked by the inability of any vaccine candidate to elicit antibodies capable of neutralizing infectivity of a variety of primary isolates (PIs) from HIV infected individuals (Wrin, T., et al., 1995, Journal of Virology 69:39; Moore, J.P., et al., 1995, AIDS 9 (suppl A), S117; Mascola, J.R., et al., 1996, Journal of Infectious Diseases 173:340.

Unfortunately, to date, researchers have been unsuccessful in producing vaccines that generate antibodies which block a wide variety of primary isolates of HIV. Primary isolates are taken directly from infected persons and subjected to only limited growth in the laboratory in primary peripheral blood lymphocytes (PB ls). Thus, primary HIV isolates are clinically more relevant than laboratory-adapted strains. Laboratory adapted strains are those which are persistently grown in established T cell lines in the laboratory (See Wrin et al., 1995, J. of Virology 69:39). Previous vaccine candidates have elicited neutralizing antibodies to laboratory-adapted strains. For example, Wrin et al., 1995, Journal of Virology 69:39, tested HIV envelope proteins produced by recombinant DNA technology derived from laboratory adapted isolates. Antibodies generated by immunization with these recombinant proteins neutralized only laboratory-adapted isolates

of the virus. Recombinant viral vectors and DNA used to produce native oligomeric envelope protein *in situ* also failed to elicit neutralizing antibodies to primary isolates. Additionally, immunogens produced by recombinant technology comprising the gp120 and soluble CD4 receptor elicited antibodies to gp120 (Gershoni, 1993, FASEB Journal 7:1185), but these antibodies were unable to neutralize primary isolates.

Neutralizing antibodies are important to prevent binding and fusion of the virus to the host cell. The action of antibodies that neutralize laboratory-adapted isolates have been analyzed. Neutralizing antibodies directed specifically to the CD4 binding domain on gp120 interfere with CD4 binding. Most neutralizing antibodies, however, interfere with steps subsequent to the binding of CD4. For example, some neutralizing antibodies inhibit interaction with the co-receptor (Trkola et al., 1998, Journal of Virology 72:1876; Wu, et al., 1996, Nature 384:179-83).

The identification of immunogens that elicit neutralizing antibodies in a host is necessary to the development of efficacious vaccines. Neutralizing antibodies must act upon not only laboratory-adapted isolates, but the clinically relevant primary isolates as well. As can be understood from the above, there remains a need for new immunogen molecules which generate antibodies that effectively neutralize a wide variety of primary isolates.

3. SUMMARY OF THE INVENTION

The present invention is based on the surprising discovery that highly effective vaccines may be constructed which present transitional fusion-related determinants to the immune system that engender unique antibodies capable of potently neutralizing a broad range of primary isolates of enveloped viruses, from worldwide locations and from different phylogenetic viral clades. The vaccine of the invention comprise as immunogens Fusion-Related Molecular Structures (FRMS) which comprise one or more fusion-related determinants. The invention is further based on the discovery that the broad and uniform neutralization of diverse primary isolates indicates that the critical determinants presented by FRMS are highly conserved and may be intimately tied to the basic functioning of the envelope protein in binding and fusion. The present invention provides the use of such FRMS in a variety of compositions and methods, including, for example, as vaccine immunogens, blood product additives, anti-viral agents, diagnostics and therapeutics.

The present invention concerns FRMS capable of eliciting neutralizing antibodies to enveloped viral pathogens. Epitopes which elicit these neutralizing antibody result from conformational changes during the process of binding and fusion of the viral envelope and host cell membranes.

In one embodiment, the complexes result from the interaction of viral proteins with at least one host cellular receptor or co-receptor and are created by co-culturing cells transformed with a nucleic acid expressing a viral protein and cells expressing host cellular receptor(s). Preferably, the cells recombinantly express the host cell receptor(s). In a further embodiment, cultures are fixed at the onset of cell-cell interaction to preserve fusion-competent immunogens. In a preferred embodiment, the fusion-related immunogens are formed as a result of the interaction of the major human immunodeficiency virus type 1 (HIV-1) envelope protein, the host cellular receptor CD4 and the host cellular co-receptor CCR5.

Vaccinating with the FRMS of the subject invention as immunogens raises an immune response to the viral pathogen and elicits the production of neutralizing antibodies in the vaccinated host. In addition to using the FRMS as a vaccine, antibodies raised to the FRMS can be used to study the molecular pathway toward fusion of the virus and host cell and to identify points of a possible antibody-mediated blockade to that pathway. Additionally, specific high-affinity "tags" may be expressed as part of the envelope protein or host cellular receptor(s) that faclilitates isolation and purification of the FRMS of the invention.

The present invention further relates to the development of monoclonal antibodies (mAb) elicited by these FRMS immunogens. Monoclonal antibodies which are capable of neutralization of primary isolates are provided. Neutralizing mAbs to functionally conserved fusion-dependent epitopes may also be useful for passive immunization in post-exposure treatments or fetus immunization.

The present invention provides an isolated molecular structure comprising an epitope formed as a result of association of (a) an envelope protein of an enveloped virus, with (b) one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins.

The present invention provides an isolated molecular structure comprising an epitope formed as a result of association of (a) an HIV envelope protein, or a mutant thereof that assembles into the viral envelope; with (b) human CD4 and a co-receptor for HIV fusion. In one embodiment, co-receptor is the chemokine receptor CCR5 or CXCR4. In another embodiment, the molecular structure is formed by association of a mutant of HIV gp41 that is fusion-defective. In another embodiment, the mutant contains one or more mutations selected from the group consisting of V2E, G10V, V570R, and Y586E. In yet another embodiment the molecular structure is formed by association of wild-type HIV envelope protein.

The present invention provides an isolated molecular structure comprising an epitope formed as a result of association of (a) a mutant envelope protein of an enveloped virus, which envelope protein in wild-type form functions in fusion of the viral envelope with a host cell membrane, and which mutant envelope protein is fusion-defective; and (b) one or more host cellular membrane proteins which function as receptors for said envelope protein.

In one embodiment, said virus is from a viral family is selected from the group consisting of Retroviridae, Rhabdoviridae, Caronaviridae, Filoviridae, Poxviridae, Bunyaviridae, Flaviviridae, Togaviridae, Orthomyxoviridae, Paramyxoviridae, and Herpesviridae. In another embodiment, the envelope protein is E1 and E2 of HCV and the cellular membrane protein is CD81. In another embodiment the molecular structure is a cross-linked cellular molecular structure. In yet another embodiment, the molecular structure is isolated from a cell lysate. In one embodiment, the cellular molecular structure comprises cells recombinantly expressing the envelope protein. In yet another embodiment, the cell lysate is from a plurality of cells comprising cells recombinantly expressing the envelope protein. In still another embodiment, the cellular molecular structure further comprises cells recombinantly expressing the one or more host cellular membrane proteins.

The present invention provides a recombinant enveloped virus, wherein said virus recombinantly expresses on its envelope a cell receptor for a native envelope protein of said virus. In one embodiment, said cell receptor is human CD4 or a co-receptor for HIV, or said virus recombinantly expresses both human CD4 and said co-receptor. In another embodiment, said suitable conditions comprise a lowering of pH. In another embodiment, the cross-linked cellular molecular structure further comprises a cross-linked viral particle of said virus, containing said envelope protein.

The present invention provides a vaccine formulation comprising an immunogenic amount of the molecular structure and a pharmaceutically acceptable carrier.

The present invention provides a monoclonal antibody to the molecular structure. In one embodiment, the antibody is labeled.

The present invention provides a purified polyclonal antiserum specific to the molecular structure.

The present invention provides a contraceptive jelly, foam, cream, or ointment comprising an amount of the antibody effective to inhibit or decrease infection by the virus.

The present invention provides a contraceptive jelly, foam, cream, or ointment comprising an amount of the antibody effective to inhibit or decrease infection by HIV.

The present invention provides a contraceptive jelly, foam, cream, or ointment comprising an amount of the antiserum effective to inhibit or decrease infection by HIV.

The present invention provides a sample of mammalian blood, to which an amount of the antibody has been added effective to inhibit or decrease infection by the virus.

The present invention provides a sample of human blood, to which an amount of the antibody has been added effective to inhibit or decrease infection by HIV. In one embodiment, said envelope protein or host cellular membrane proteins further comprises an affinity tag. In another embodiment, said envelope protein, CD4, or co-receptor further comprises an affinity tag. In yet another embodiment, the antibody is labeled.

The present invention provides a kit comprising in one or more containers a labeled monoclonal antibody to the molecular structure.

The present invention provides a kit comprising in one or more containers a labeled monoclonal antibody to the molecular structure. In one embodiment, the kit comprises the molecular structure in a separate container.

The present invention provides a cell line that recombinantly expresses an envelope protein of an enveloped virus that functions in fusion of the viral envelope with a host cell membrane, or a mutant form of said envelope protein that is fusion-defective, which cell line expresses one or more cellular membrane proteins that function as receptors for said envelope protein. In one embodiment, said one or more proteins that function as receptors are recombinantly expressed.

The present invention provides a cell line that recombinantly expresses HIV gp160, which cell line expresses CD4 and a co-receptor for HIV; said cell line lacking a functional protease that cleaves gp160 to produce gp120 and gp41.

The present invention provides a method of treating or preventing infection by a virus in a subject comprising administering to the subject an immunogenic amount of the molecular structure effective to treat or prevent infection by the virus. In one embodiment, the subject is a human. In another embodiment, the subject is a domestic animal.

The present invention provides a method of treating or preventing infection by HIV in a human comprising administering to the human an immunogenic amount of the molecular structure effective to treat or prevent infection by HIV.

The present invention provides a method of treating or preventing infection by a virus in a subject comprising administering to the subject an amount of the monoclonal antibody effective to treat or prevent infection by the virus.

The present invention provides a method of treating or preventing infection by HIV in a human comprising administering to the human an amount of the monoclonal antibody effective to treat or prevent infection by HIV. In one embodiment, said human has a high risk of HIV infection. In one embodiment, the method is for treatment of AIDS in said human.

The present invention provides a method for treating or preventing infection by HIV in a human fetus comprising administering to a pregnant human containing said fetus an amount of the monoclonal antibody effective to treat or prevent infection by HIV in said fetus.

The present invention provides a method of inhibiting infection by a virus in a sample of blood comprising contacting said sample of blood with an amount of the monoclonal antibody effective to inhibit infection by said virus.

The present invention provides a method of inhibiting infection by HIV in a sample of human blood comprising contacting said sample of human blood with an amount of the monoclonal antibody effective to inhibit infection by HIV.

The present invention provides a method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody effective to inhibit infection by said virus.

The present invention provides a method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody effective to inhibit infection by HIV.

The present invention provides a method for monitoring the production of antibody to the molecular structure in a subject previously administered an amount of the molecular structure comprising isolating from said subject a sample comprising serum; and detecting the presence of any antibodies to the molecular structure in said serum. In one embodiment, said detecting is carried out by a method comprising performing a competitive immunoassay with labeled antibody to the molecular structure.

The present invention provides a method of producing an immunogen for use in a vaccine for the treatment or prevention of infection by a virus comprising the following steps in the order stated: (a) contacting an envelope protein or chimeric form thereof of an enveloped virus, which envelope protein functions in fusion of the viral envelope with a cell membrane, or a mutant form or chimeric form thereof of said envelope protein that is fusion-defective, with one or more cell proteins or chimeric forms thereof that function as receptors for said envelope protein; (b) exposing said envelope protein or chimeric form thereof or mutant form or chimeric form thereof, and said one or more host cell proteins or chimeric forms thereof, to a cross-linking agent; and (c) isolating a cross-linked structure

comprising said envelope protein or chimeric form thereof or mutant form or chimeric form thereof.

The present invention provides a method of producing an immunogen for use in a vaccine for the treatment or prevention of infection by HIV comprising the following steps in the order stated: (a) co-culturing a first cell recombinantly expressing HIV envelope protein or a chimeric form thereof, or a mutant form or chimeric form thereof of said envelope protein that is fusion-defective, with a second cell that expresses (i) human CD4 or a chimeric form thereof, and (ii) a co-receptor for HIV or a chimeric form thereof; (b) exposing said envelope protein or chimeric form thereof or mutant form or chimeric form thereof, and said CD4 or chimeric form thereof and co-receptor or chimeric form thereof, to a cross-linking agent; and (c) isolating a cross-linked structure comprising said envelope protein or chimeric form thereof or mutant form or chimeric form thereof. In one embodiment, said virus is HIV and said host cell proteins are human CD4 and a co-receptor for HIV. In one embodiment, said second cell recombinantly expresses CD4 or said co-receptor or both CD4 and said co-receptor or chimeric forms of any of the foregoing.

In other embodiment, said first and second cell are the same cell type. In yet another embodiment, said envelope protein or chimeric form thereof or mutant form or chimeric form thereof is present on a viral particle or virus-like particle. In still another embodiment, said cross-linked structure is a cross-linked cellular complex.

In another embodiment, the virus is selected from the group consisting of Retroviridae, Rhabdoviridae, Coronaviridae, Filoviridae, Poxviridae, Bunyaviridae, Flaviviridae, Togaviridae, Orthomyxoviridae, Paramyxoviridae, and Herpesviridae.

In another embodiment, said contacting step occurs by infecting cells expressing said host cell proteins with said virus expressing said envelope protein or chimeric form thereof or mutant form or chimeric form thereof. In another embodiment, a chimeric form of said envelope protein or one of said host cell proteins is contacted, said chimeric form comprising an affinity tag. In another embodiment, a chimeric form of said envelope protein or CD4 or said co-receptor is contacted, said chimeric form comprising an affinity tag.

The present invention provides a method of producing an immunogen for use in a vaccine for the treatment or prevention of infection by HIV comprising the following steps in the order stated: (a) co-culturing a first cell recombinantly expressing HIV envelope protein or a chimeric form thereof, or a mutant form or chimeric form thereof of said envelope protein that is fusion-defective, with a second cell that expresses (i) human CD4 or a chimeric form thereof, and (ii) a co-receptor for HIV or a chimeric form thereof wherein at least one of said chimeric forms comprising an affinity tag is expressed; (b) lysing said co-cultured cells to form a cell lysate under non-denaturing conditions; and (c)

isolating from said cell lysate a molecular structure comprising said envelope protein or chimeric form thereof or mutant form or chimeric form thereof by a method comprising contacting said cell lysate with a binding partner to said affinity tag and recovering a molecular structure bound to said affinity tag.

The present invention provides a cross-linked structure that is the product of the method.

The present invention provides a monoclonal antibody to the structure of that neutralizes *in vitro* the following primary isolates of HIV: 92US657, 92US660, 92RW023, 93IN101, 92UG035, and 92TH023.

The present invention provides a contraceptive, jelly, foam, cream or ointment comprising an amount of the antibody to inhibit or decrease infection by HIV.

The present invention provides a method of treating or preventing infection by a virus in a subject comprising administering to the subject an immunogenic amount of the structure effective to treat or prevent infection by the virus.

The present invention provides a method of treating or preventing infection by HIV in a human comprising administering to the human an immunogenic amount of the molecular structure effective to treat or prevent infection by HIV.

The present invention provides a method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody effective to inhibit infection by HIV.

The present invention provides a method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody effective to inhibit infection by said virus.

The present invention provides a method of screening a molecular structure for vaccine efficacy comprising immunizing a transgenic non-human mammal with the molecular structure, wherein said transgenic non-human mammal expresses from one or more transgenes both human CD4 and a co-receptor for HIV, and detecting any neutralizing antibodies to HIV that are produced by said mammal.

The present invention provides a method of screening a molecular structure for vaccine efficacy comprising immunizing a transgenic non-human mammal with the molecular structure, wherein said transgenic non-human mammal expresses from one or more transgenes said one or more host cellular membrane proteins; and detecting any neutralizing antibodies to said virus that are produced by said mammal. In one embodiment, the mammal is a mouse. In another embodiment, said first cell recombinantly expresses HIV envelope protein or a chimeric form thereof.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Neutralization of the homologous 168P PI virus by FC and FI vaccine sera. Transgenic mice (hu CD4+, hu CCR5+, mouse CD4+) were immunized with FC immunogen (COS-env with U87-CD4-CCR5; squares; n = 3 mice) or with cell controls (U87-CD4-CCR5 cells alone or cocultured with mock-transfected COS cells; circles n = 3 mice). Unimmunized mice were also used (triangles; n = 2 mice). Sera were tested for neutralization of 168P using U87-CD4 cells expressing either CXCR4 (black symbols) or CCR5 (white symbols). Data represent averages of three to six neutralization assays using serum obtained 2 weeks following second and third immunization.

Figure 2A-B. Neutralization of P168 by FC but not FI, vaccine sera. (A) Transgenic mice were immunized with FC immunogen (black squares; n = 4), FI immunogens (COS-env with U87 cells; gray circles n = 4; COS-env with U87-CD4 cells, gray diamonds, n = 3; COS-env with sCD4, white diamonds, n = 2; COS-env with U87-CD4-CCR5 cells, each fixed separately prior to mixing for immunization, gray squares, n = 2), or mock-transfected cos cell immunogen (cocultured with U87-CD4-CCR5 cells, white circles, n = 2). Unimmunized mice (white triangles, n = 2) were also used. Neutralization was independent of specific co-receptor use and data here represent averages of three to six neutralization assays in U87-CD4-CXCR4 or -CCR5 cells. In some cases, sera from all animals within each experimental group were pooled. (B) Neutralization of P168 by FC but not FI, vaccine sera. Neutralization of the homologous 168P PI virus in human PBL (lymphocyte) culture. PBLs were isolated, stimulated with phytohemagglutinin, and grown in the presence of interleukin-2; neutralization was determined. HIV p24 antigen was determined after 5 days of culture by ELISA (Coulter Corporation) and values were normalized to the virus control (36 ng/ml). Asterisks indicates p24 antigen levels below the limit of detection at the dilution used in the ELISA.

Figure 3A-B. FC vaccine serum does not neutralize pseudotyped HIV virions bearing amphotropic MLV envelope protein (A) or primary SIVmac251 (B). For HIV bearing an amphotropic MLV envelope protein (ampho MLV pseudotype) neutralization sensitivity with pooled FC and FI antisera was determined in U87-CD4-CXCR4 cells. For primary isolate SIVmac251 neutralization was determined in U87-CD4-CCR5 cells. Symbols are: FC Immunogen (black squares) and FI immunogen (Cos-env + U87 cells, gray circles).

Figure 4A-B Neutralization of TCLA 168C virus by FI vaccine sera. Neutralization sensitivity of the 168P PI virus (A) and its TCLA derivative 168C (B) were tested in U87-

CD4-CXCR4 cells with pooled sera: FC immunogen (black squares), FI immunogens (COS-env + U87 cells, gray circles; COS-env + U87-CD4 cells, gray diamonds; COS-env +sCD4, white diamonds), and mock-transfected cell controls (white circles).

Figure 5. Neutralization of diverse PI viruses from clades A-E. Primary isolates were expanded in human PBLs, and neutralization was determined in permissive U87-CD4-CCR5 (or -CXCR4) cells with pooled sera: FC immunogens (black squares), FI immunogens (COS-env + U87 cells, gray circles; COS-env + U87-CD4, gray diamonds; COS-env + sCD4, white diamonds, and mock transfected cell controls (white circles). Viral biotype is indicated in lower right corner as SI or NSI where available. Viral isotype is indicated in lower corner of each graph.

Figure 6. Adsorption of PI virus neutralization activity by formaldehyde-fixed COS-env cells. FC vaccine serum was repeatedly incubated with formaldehyde-fixed COS-env cells (gray squares) or control COS cells (white squares). Serum obtained prior to FC immunization was similarly adsorbed (gray and white circles, respectively). The starting FC and preimmunization sera are indicated as black squares or black circles, respectively. Sear were tested for neutralization of 168P using U87-CD4-CXCR4 cells.

Figure 7. PI virus neutralization activity is not adsorbed by intact U87-CD4-CCR5 cells. Pooled FC vaccine serum was incubated with U87-CD4 CCR5 cells (while squares) or in an empty microculture well (mock, black squares). Pooled FI serum (COS-env + U98-CD4) was similarly treated (white and black circles, respectively). Sera were tested for remaining neutralization of 168P with U87-CD4-CCR5 cells.

Figure 8. Neutralizing activity of two HIV Clade B primary isolates with hybridoma supernatants.

Figure 9. Serum titers of mice immunized with fusion-competent vaccine immunogens. White square represents fusion-competent (FRMS) immunogen (env+with CD4/CCR5); black circles represent immunogen from cells expressing envelope protein immunogen and cells expressing neither CD4 nor CCR5 (env); white circles represent immunogen from cells expressing envelope protein and cells expressing CD4 immunogen (env+CD4); white triangle represent cell controls expressing CD4 and CCR5 immunogen (CD4/CCR5).

Figure 10. Cross-neutralization of the 320SI primary virus isolate by antibodies obtained from mice immunized with a fusion-competent immunogen complex of the subject

invention. Black circles represent fusion-competent (FRMS) immunogen (env with CD4/CCR5); white squares represent immunogen from cells expressing envelope protein (env); white circles represent immunogen from cells expressing envelope protein and CD4 (env + CD4).

Figure 11. Envelope protein co-purified with CD4-Spep and CCR5-Spep detected by Western blot analysis. a) 168P envelope protein isolated following co-culture with cells expressing CD4-Spep b) 168P envelope protein isolated following co-culture with cells expressing CD4 and CCR5-Spep c) 168P envelope protein was undetectable following co-culture with mock-transfected cells.

Figure 12. Co-culture of BSC40 cells infected with rV-168Penv and rV-CD4/CCR5 24 hr post infection.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns fusion-related molecular-structures (FRMS) capable of eliciting neutralizing antibodies to enveloped viral pathogens. The FRMS of the invention comprise epitopes formed as a result of the association of one or more cellular molecules with one or more viral envelope molecules. The process of viral envelope fusion with a cell membrane is initiated following binding of a viral envelope protein to specific cell receptors and/or co-receptors on the host cell and involves fusion of the viral envelope with surface or internal cell membranes. Fusion is driven by conformational changes which occur within the envelope-receptor/co-receptor complex. As not intended to be limited by a particular mechanism, it is believed that the conformational changes that occur during fusion reveal new and unique immunogenic epitopes for neutralizing antibodies. The fusion-related molecular structures of the subject invention mimic the conformational intermediates formed during fusion. The FRMS of the invention may be used for a variety of purposes including but not limited to those presented in Sections 5.5 and 5.7, herein. For example, in a preferred embodiment of the invention, the FRMS when used to vaccinate host animals elicits neutralizing antibodies to the infecting virus, and thus provide novel effective components of vaccine formulations of the invention, for treatment, or prevention of viral infection and its undesirable consequences.

Accordingly, the present invention provides an isolated molecular structure comprising an epitope formed as a result of association of (a) an envelope protein of an enveloped virus, with (b) one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular

membrane proteins. As used herein a cellular membrane protein includes proteins of the cell surface membrane and internal membranes (including but not limited to the endoplasmic reticulum membrane). Such cellular membrane protein may be an integral membrane protein (e.g., a trans-membrane protein), or may be attached to the membrane by an attached lipid (e.g., fatty acid chain or prenyl group) or by an oligosaccharide (e.g., to a phopholipid, phosphatidylinositol), or the cellular membrane protein may also be associated with the membrane by non-covalent interactions (e.g., by association with an integral membrane protein).

The present invention also provides an isolated molecular structure comprising an epitope formed as a result of association of (a) a mutant envelope protein of an enveloped virus, which envelope protein in wild-type form functions in fusion of the viral envelope with a host cell membrane, and which mutant envelope protein is fusion-defective; and (b) one or more host cellular membrane proteins which function as receptors for said envelope protein.

In a specific embodiment, the present invention provides an isolated molecular structure comprising an epitope formed as a result of association of (a) an HIV envelope protein, or a mutant thereof that assembles into the viral envelope; with (b) human CD4 and a co-receptor for HIV fusion. In one embodiment, the co-receptor is the chemokine receptor CCR5 or CXCR4. In another specific embodiment, the molecular structure is formed by association of a mutant of HIV gp41 that is fusion-defective. In yet another embodiment, the mutant contains one or more mutations selected from the group consisting of V2E, G10V, V570R, and Y586E. In another embodiment the molecular structure is formed by association of wild-type HIV envelope protein. In another specific embodiment, the envelope protein is E1 and E2 of Flavivirus HCV and the cellular membrane protein is CD81.

The present invention also provides a method of producing an immunogen for use in a vaccine for the treatment or prevention of infection by a virus comprising the following steps in the order stated: (a) contacting an envelope protein or chimeric form thereof of an enveloped virus, which envelope protein functions in fusion of the viral envelope with a cell membrane, or a mutant form or chimeric form thereof of said envelope protein that is fusion-defective, with one or more cell proteins or chimeric forms thereof that function as receptors for said envelope protein; (b) exposing said envelope protein or chimeric form thereof or mutant form or chimeric form thereof, and said one or more host cell proteins or chimeric forms thereof, to a cross-linking agent; and (c) isolating a cross-linked structure comprising said envelope protein or chimeric form thereof or mutant form or chimeric form thereof. In one embodiment, said virus is HIV and said host cell proteins are human CD4 and a co-receptor for HIV.

In a specific embodiment, the present invention also provides a method of producing an immunogen for use in a vaccine for the treatment or prevention of infection by HIV comprising the following steps in the order stated: (a) co-culturing a first cell recombinantly expressing HIV envelope protein(s) or a chimeric form thereof, or a mutant form or chimeric form thereof of said envelope protein that is fusion-defective, with a second cell that expresses (i) human CD4 or a chimeric form thereof, and (ii) a co-receptor for HIV or a chimeric form thereof; (b) exposing said envelope protein or chimeric form thereof or mutant form or chimeric form thereof, and said CD4 or chimeric form thereof and co-receptor or chimeric form thereof, to a cross-linking agent; and (c) isolating a cross-linked structure comprising said envelope protein or chimeric form thereof or mutant form or chimeric form thereof.

In one specific embodiment, said second cell recombinantly expresses CD4 or said co-receptor or both CD4 and said co-receptor or chimeric forms of any of the foregoing. In another specific embodiment, the present invention provides a method of producing an immunogen for use in a vaccine for the treatment or prevention of infection by HIV comprising the following steps in the order stated: (a) co-culturing a first cell recombinantly expressing HIV envelope protein or a chimeric form thereof, or a mutant form or chimeric form thereof of said envelope protein that is fusion-defective, with a second cell that expresses (i) human CD4 or a chimeric form thereof, and (ii) a co-receptor for HIV or a chimeric form thereof wherein at least one of said chimeric forms comprising an affinity tag is expressed; (b) lysing said co-cultured cells to form a cell lysate under non-denaturing conditions; and (c) isolating from said cell lysate a molecular structure comprising said envelope protein or chimeric form thereof or mutant form or chimeric form thereof by a method comprising contacting said cell lysate with a binding partner to said affinity tag and recovering a molecular structure bound to said affinity tag.

5.1. FORMATION OF THE FRMS

The present invention relates to FRMS which comprise an epitope formed as a result of the association of one or more viral envelope molecule(s) and one or more host cell molecule(s). The FRMS of the invention encompass structures resulting from the association of at least one viral envelope protein and at least one host cell protein. For example, a FRMS of the invention may encompass fusion-competent complexes (such as complexes formed during fusion events of the viral envelope with a cellular membrane), fusion-defective complexes (such as those formed during pre-fusion events between a fusion-defective viral envelope and a cellular membrane) as well as complexes formed by the association of one or more viral envelope molecules with one or more cellular receptor

and/or co-receptor for the virus. In the case of HIV, the FRMS result from the association of HIV envelope protein with CD4 and a chemokine receptor.

The FRMS of the invention, thus comprise epitopes capable of eliciting neutralizing antibodies to the virus.

The FRMS of the invention are formed by a variety of methods as described herein. In a preferred embodiment, cells expressing one or more components whose association results in the FRMS are cultured to form the FRMS. All the components may be expressed on one cell, or a first component may be expressed on one cell, and a second component may be expressed on a different cell, and a third component (if any) may be expressed on one of the foregoing cells or a different cell. Alternatively, one of the cells may be replaced by a viral particle or pseudovirus or recombinant virus or virus-like particle containing the component(s) on its surface. The components (viral envelope protein, host cell receptor, and/or any necessary host cell co-receptor) may each be endogenously or recombinantly expressed by the cells. Thus, FRMS may be formed by the interaction of endogenous molecules, exogenous molecules (*e.g.* recombinantly expressed) or any combination thereof.

In yet another embodiment of the invention, a soluble form of a viral envelope protein of a envelope virus is used in the formation of a FRMS of the invention. For example, in one embodiment the soluble envelope protein may be added t o host cells in vitro which host cells express the receptor(s) for the virus. Thus the association of the soluble viral envelope protein and host cell receptor(s) results in the formation of a FRMS.

In other embodiment of the invention one or more soluble cell recptor(s) are used in formation of the FRMS. In one embodiment, for example, soluble cellular receptor(s) whose association with the viral envelope protein(s) results in the formation of a FRMS, may be used in the formation of a FRMS by contacting the viral envelope protein(s) with said soluble cell receptor(s). In another specific embodiment, all of the components whose association result in the formation of the FRMS are in a soluble form and the FRMS is reconstitutional in vitro. In this embodiment, the reconstitution of a FRMS may optionally be aided by the addition of a mAb to the FRMS.

In yet other embodiments of the invention the FRMS is forced upon a decrease in pH. For example, in some embodiments, such pH-medicated cell fusion allows for the formulation of the FRMS for enveloped viruses which cellular membrane proteins are located in internal cellular membranes.

5.1.1. ENDOGENOUS EXPRESSION OF COMPONENTS OF THE COMPLEX

The cellular components involved in the formation of a FRMS for a particular virus include the cell receptors and/or co-receptors for the particular virus. In a specific embodiment, at least some of these components are endogenously expressed by the cell. Thus, any cell known in the art which expresses a cellular component of the FRMS may be used in the formation of a FRMS.

Cells expressing native cellular receptors and/or co-receptors for viral proteins can be used. For example, as described herein, human CD4 is known in the art as a cellular receptor for HIV. In one embodiment of the invention, cells endogenously expressing CD4 are used in the formation of a FRMS.

Cells endogenously expressing a component of the complexes of the invention may comprise one or more of the cellular components used to form the FRMS. For example, cells may be used which express all of the required cellular components of the FRMS or cells may be used which do not express all of the required cellular components. For example, in the case of an HIV FRMS, any cell which expresses both CD4 and an HIV co-receptor, such as CCR5 may be used in the formation of an HIV FRMS. Alternatively, any cell which endogenously express CD4 but does not endogenously express an HIV co-receptor, in this embodiment, and as discussed below, components of the complex which are not endogenously expressed may be introduced into the cell and recombinantly expressed. In this embodiment, additional cellular components of the complex are introduced to the cell such that the cell expresses both endogenous and exogenous cellular components. In one specific embodiment of the invention, the co-receptor CXCR4 is predominantly located on T cell lines. In another specific embodiment of the invention the co-receptor CCR5 is predominantly located on macrophage cells. In one specific embodiment of the invention, the co-receptor CXCR4 is predominantly located on T cell lines. In another specific embodiment of the invention the co-receptor CCR5 is predominantly located on macrophage cells. Exogenous components may be introduced into the cell by any method known in the art, including those described in Section 5.1.2. below.

In yet another embodiment of the invention, a cell transformed by means of a viral gene or genome integration, which expresses a viral component of the FRMS may be used in the formation of a fusion related complex. In this manner, the cell may be said to endogenously express a viral component important in the construction of an FRMS. Such virally-transformed cells may also endogenously express one or more cellular components of the FRMS. Alternatively, viral particles may be used as a source of endogenous expression of viral components of a FRMS.

5.1.2. RECOMBINANT EXPRESSION OF COMPONENTS IMPORTANT IN THE FORMATION OF THE FRMS

Any of the components important in the construction of a FRMS or fragments, analogs or derivatives thereof may be introduced into a cell such that the exogenous component is expressed in the cell.

The present invention encompasses expression systems, both eucaryotic and procaryotic expression vectors, which may be used to express a component important in the formation of a FRMS or fragment, analog or derivative thereof of the present invention.

In other embodiments of the invention, the viral envelope protein is expressed in a host cell. In this embodiment, the exogenous viral envelope protein may be introduced into the cell by transfection or viral vectors as described herein, or by infection with the live or attenuated virus comprising the viral envelope protein. Thus, viral infection can lead to production of a viral envelope protein in preferred embodiments, the expression of a viral envelope protein on the cell membrane is enhanced by the modification (*e.g.* by recombinant means) of the viral envelope protein so as to increase its expression on the cell surface, or other cell membrane. For example, targeting signals are known in the art that enhance delivery of cellular proteins to particular subcellular compartments or locations. For example, an endoplasimic reticulum signal, KDEL, (one letter amino acid codes) could be engineered into the viral envelope protein to promote targetry of the protein to the ER when a cell is infected with the virus.

The invention also provides a cell expressing the viral and cellular components important for the formation of a FRMS. In one embodiment, a cell is constructed to express the cell receptor and/or co-receptor for a virus and to recombinantly express the viral envelope protein. Such cell may endogenously express the host cell receptors and/or co-receptors for the virus or recombinantly express one or both of such protein(s). In embodiments wherein viral and cellular proteins are recombinantly expressed, the nucleic acids encoding such proteins may be expressed from different vectors or from a single vector (*e.g.*, wherein a vector comprises multiple genes operably linked to a single promoter). Thus, in this embodiment all of the components necessary for the formation of a FRMS are expressed on an individual cell. In a specific embodiment, a cell is constructed to recombinantly express CD4, a co-receptor CCR5 or CXCR4 and HIV gp160. Alternatively, gp120 and gp41 may be used in place of gp160. In a preferred embodiment, when an individual cell is constructed to express gp160, CD4 and a co-receptor for HIV-1 the viral envelope protein is also constructed such that it is lacking the native protease site responsible for cleavage of gp160 to gp120 and gp41. In this embodiment an alternative protease site is constructed into the viral envelope protein which protease is not native to the host cell (such as a bacterial protease). Formation of the FRMS

is triggered on the cell expressing the three proteins, by contacting the cell with the non-native protease (such as by adding an amount effective to induce cleavage, to the cell media). A variety of proteases and protease sites are known in the art and may be used in accordance with the invention.

The nucleotide sequence coding for a viral envelope protein or cellular receptor protein of the invention or a functionally active analog or fragment or other derivative thereof, can be inserted into an appropriate expression vector, *e.g.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence and/or for carrying out the methods of the invention.

The invention encompasses the use of DNA expression vectors and/or viral vectors that contain coding sequences of a component of FRMS or analog, derivative or fragment thereof operatively associated with a regulatory element that directs expression of the coding sequences and genetically engineered host cells that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell.

The DNA expression vectors and viral vectors containing the nucleic acids encoding a component important in the formation of a FRMS of the present invention may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the expression vectors and viral vectors of the invention by expressing nucleic acid containing sequences encoding a component important in the formation of a FRMS or analog, derivative or fragment thereof are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing gene product coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and genetic recombination. See, for example, the techniques described in Sambrook, *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Press, N.Y.; and Ausubel, *et al.*, 1989-1999, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y., both of which are incorporated herein by reference in their entirety. Alternatively, gene product sequences may be chemically synthesized using, for example, oligonucleotide synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety. Gene expression may be regulated by a variety of methods known in the art including but not limited to those presented in Mizuno, T. et al., 1984, Proc. Natl. Acad Sci USA. 81(7):1966-70.

The nucleic acids or DNA encoding one or more components which result in the formation of the FRMS may be introduced into a cell may be accomplished by a variety

of methods, such as liposomes, electroporation, transfection, viral vectors, bacteriophage, etc.

In one embodiment, nucleic acids encoding one or more components important in the formation of a FRMS is accomplished by packaging plasmid DNA comprising the nucleic acids that code for the component(s) into liposomes or by complexing the plasmid DNA comprising nucleic acids that code for the component(s) with lipids or liposomes to form DNA-lipid or DNA-liposome complexes. The liposome is composed of cationic and neutral lipids commonly used to transfect cells *in vitro*. The cationic lipids complex with the plasmid DNA and form liposomes.

Cationic and neutral liposomes are contemplated by this invention. Cationic liposomes can be complexed with the a negatively-charged biologically active molecule (*e.g.*, DNA) by mixing these components and allowing them to charge-associate. Cationic liposomes are particularly useful with a nucleic acid because of the nucleic acids negative charge. Examples of cationic liposomes include lipofectin, lipofectamine, lipofectace and DOTAP (Hawley-Nelson et al., 1992, Focus 15(3):73-83; Felgner et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 84:7413; Stewart et al., 1992, Human Gene Therapy 3:267-275). Commercially available cationic lipids are also available, *e.g.*, dimethyldioctadeclammonium bromide (DDAB); a biodegradable lipid 1, 2-bis(oleoyloxy)-3-(trimethylammonio) propane (DOTAP); these liposomes may be mixed with a neutral lipid, *e.g.*, L-α dioleoyl phosphatidylethanolamine (DOPE) or cholesterol (Chol), two commonly used neutral lipids for systemic delivery. DNA:liposome ratios may be optimized using the methods used by those of skill in the art (*see e.g.*, Nicolau et al., 1987, Methods Enzymol. 149:157) and can readily be utilized herein by one of ordinary skill in the art to encase the complex of this invention.

In yet another embodiment of the present invention, the plasmid DNA coding for the genes or nucleic acids encoding one or more components resulting in the formation of a FRMS of the invention may be delivered via polycations, molecules which carry multiple positive charges and are used to achieve gene transfer *in vitro, in vivo* and *ex vivo*. Polycations, such as polyethilenimine, may be used to achieve successful gene transfer (*see e.g.*, Boletta et al., 1996, J. Am. Soc. Nephrol. 7:1728).

Recombinant viruses can also be used to deliver the components whose association results in the FRMS. Cells infected by these viruses or transformed by the viral genome will thus express the desired components. Thus, in another embodiment of the present invention, either a live recombinant vector or an inactivated recombinant viral vector expressing one or more component(s) described herein can be engineered. In this regard, a variety of viruses may be genetically engineered to express a component important

in the formation of a FRMS. In some case, it may be desired that the recombinant viruses are either cold adapted, temperature sensitive, or attenuated.

In accordance with the present invention, a wide variety of viruses and viral vectors may be used to deliver the nucleotide sequences encoding one or more component(s) important to the formation of a FRMS of the present invention, a few examples of which are described below.

Retroviral vectors are commonly used to deliver genes to host cells. Retroviral vectors are extremely efficient gene delivery vehicles that cause no detectable harm as they enter the cells. The retroviral nucleic acid may integrate into host chromosomal DNA allowing for long-term persistence and stable transmission to future progeny, such a vector would be useful for the delivery of one or more component(s) which result in the formation of a FRMS. An example of an appropriate retroviral vector are lentiviruses which have the advantage of infecting and transducing non-dividing cells. In such an embodiment, a lentiviral vector encoding a packagable RNA vector genome and operably linked to a promoter in which all the functional retroviral auxiliary genes are absent, is used to transfer the DNA encoding a component important in the formation of a FRMS of the present invention. Examples of such vectors are described in WO 98/17815, WO 98/17816 and WO 98/17817, each of which is incorporated herein by reference in their entirety.

In yet another embodiment, non-integrating viral vectors which infect and transduce non-dividing cells, such as adenoviral vectors may be used to deliver a component important in the formation of a FRMS. Adenoviral vectors have several advantages since such vectors avoid risks associated with permanently altering the host cell genome or promoting insertional mutagenesis. Adenoviruses are one of the best developed non-integrating viral vectors and can be used to transfer expression cassettes of up to 75 kb. Recombinant adenoviruses can be produced at very high titers is highly infectious and efficiently transfer genes to a wide variety of non-replicating and replicating cells and is further ideal for *in vivo* mammalian gene transfer.

Adenovirus-based vectors are relatively safe and can be manipulated to encode the desired component(s) and at the same time to be inactivated in terms of its ability to replicate in a normal lytic viral life cycle. Adenovirus has a natural tropism for airway epithelia. Therefore, adenovirus-based vectors are particularly preferred for epithelial delivery applications. In a particular embodiment, the adenovirus-based gene therapy vector comprises an adenovirus 2 serotype genome in which the Ela and the Elb regions of the genome, which are involved in early stages of viral replication have been deleted and replaced by nucleotide sequences of interest. In a further embodiment, the adenovirus --based gene therapy vector contains only the essential open reading frame

(ORF3 or ORF6 of adenoviral early region 4 (E4) and is deleted of all other E4 open reading frames, or may additionally contain deletions in the E3 regions (*see e.g.* U.S. Patent No. 5,670,488, incorporated herein by reference in its entirety). In another embodiment, the adenovirus-based therapy vector used may be a pseudo-adenovirus (PAV), which contain no harmful viral genes and a theoretical capacity for foreign material of nearly 36 kb.

In another embodiment, adeno-associated virus (AAV) systems may be used to deliver the component important in the formation of a FRMS of the present invention. AAV has a wide host range and AAV vectors have currently have been designed which do not require helper virus. Examples of such AAV vectors are described in WO 97/17458.

Vaccinia viral vectors may be used in accordance with the present invention, as large fragments of DNA are easily cloned into its genome and recombinant attenuated vaccinia variants have been described (Meyer, et al., 1991, J. Gen. Virol. 72:1031-1038). In one embodiment of the invention, a vaccinia viral vector is used to deliver the components important to the formation of an FRMS such that the components are expressed in the recombinant vaccinia infected cell. In a specific embodiment, the components are human CD4, a co-receptor (e.g., CCR5 or CXCR4), and the HIV envelope protein. In this specific embodiment, infection of a cell with the recombinant vaccinia results in the expression of the components and formation of an HIV FRMS.

In another specific embodiment, two different recombinant vaccinia viruses are constructed such that the first virus encodes the HIV env protein, and the second virus encodes human CD4 and a chemokine receptor. In this embodiment, the first virus is used to infect a first population of cells such that the infection results in the expression of the HIV env protein. The second virus is used to infect a second population of cells such that the infection results in the expression of CD4 and the chemokine receptor. Co-culturing of the first infected population of cells with the second infected population of cells results in the formation of an HIV FRMS. In a further embodiment, the co-cultured cells may be cross-linked.

Orthomyxoviruses, including influenza; Paramyxoviruses, including respiratory syncytial virus and Sendai virus; and Rhabdoviruses may be engineered to express mutations which result in attenuated phenotypes (*see* U.S. Patent Serial No. 5,578,473, issued November 26, 1996 incorporated herein by reference in its entirety). These viral genomes may also be engineered to express foreign nucleotide sequences, such as a component important in the formation of a FRMS of the present invention (*see* U.S. Patent Serial No. 5,166,057, issued November 24, 1992 incorporated herein by reference in its entirety).

Reverse genetic techniques can be applied to manipulate negative and positive strand RNA viral genomes to introduce mutations which result in attenuated

phenotypes, as demonstrated in influenza virus, measles virus, Sindbis virus and poliovirus (*see* Palese et al., 1996, Proc. Natl. Acad. Sci. USA 93:11354-11358). These techniques may also be utilized to introduce foreign DNA, *i.e.*, encoding a component or protein important in the formation of a FRMS to create recombinant viral vectors to be used in accordance with the present invention. In addition, attenuated adenoviruses and retroviruses may be engineered to express a component important in the formation of a FRMS. Therefore, a wide variety of viruses may be engineered to deliver a component important in the formation of a FRMS of the present invention.

Bacteriophage may be used to specifically infects a bacterial cell (Soothill, J.S., 1992, J. Med. Microbiol. 37:358-261).

In the viral vectors of the present invention, the non-viral DNA (e.g., encoding a cell receptor) or non-native viral DNA (e.g., encoding a viral envelope protein) can encode any component important in the formation of a FRMS.

Thus, one skilled in the art would realize that viral envelope protein(s), cell receptor(s), and/or co-receptor(s) used to construct the FRMS of the subject invention can be provided to cells by virions, virus-infected cells or chemically/genetically inactivated virions, viral vectors, viral replicons (*i.e.* non-replicating viral constructs, *e.g.* VEE replicons), virus-like particles produced by recombinant DNA methods or as naked DNA.

As herein above, recombinant expression systems may employ regulatory elements including not limited to, inducible and non-inducible promoters, enhancers, operators. Any of the methods previously described for the insertion of DNA or nucleic acid fragments into a vector may be used to construct expression vectors containing a gene or chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques. In general, expression of a nucleic acid sequence encoding a protein or peptide fragment may be regulated by a second nucleic acid sequence so that the protein or peptide is expressed in a host cell transformed with the recombinant DNA molecule. For example, expression of a viral envelope protein may be controlled by any promoter/enhancer element known in the art. A promoter/enhancer may be homologous (*i.e.* native) or heterologous (*i.e.* not native). Promoters which may be used to control the expression of a protein include, but are not limited to, the SV40 early promoter region (Benoist et al., 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213), the cauliflower mosaic virus 35S RNA

promoter (Gardner et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120), promoter elements from yeast or other fungi such as the Gal4-responsive promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); a gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), an immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in erythroid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to a nucleic acid encoding a viral or cellular protein or chimeric protein and one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene). In another embodiment, two or more promoters may be used to direct expression of two or more genes within a single plasmid.

In a specific embodiment the CMV immediate early (IE) promoter is used to direct expression of a nucleic acid encoding a viral or cellularprotein or chimeric protein.

Promoters can be inducible, or constitutive. Expression from certain promoters can be elevated in the presence of certain inducers; thus, for example, expression of the genetically engineered protein chimeras may be controlled. Inducible promoters may be used to control expression of the proteins of the invention, such that the protein is produced only in the presence of the inducer.

The present invention also provides methods for stable expression of the components which result in the formation of an FRMS of the invention. For long term, high-yield production of recombinant proteins, stable expression is possible. For example, cell lines which stably express one or more cellular receptors and co-receptors for the virus, may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter sequences, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. For example, following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection foci (*e.g.*, by stably integrating the plasmid into their chromosomes) and allows cells to and grow to form which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines. This method may advantageously be used to engineer cell lines which express the selected gene products. Such cell lines would be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the selected gene product.

A number of selection systems may be used in generating stably-expressing cell lines, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al., 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: DHFR, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan et al., 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

5.1.3. CELLS EXPRESSING COMPONENTS WHOSE ASSOCIATION RESULTS IN FRMS

The present invention encompasses the expression of component(s) whose association results in the formation of the FRMS. In several embodiments of the invention, expression may be in primary cells, animal cells, and insect cell lines. In accordance with the present invention, a variety of primary or secondary cells or cell strains may be used including but not limited to cells isolated from skin, bone marrow, liver, spleen, pancreas, kidney, adrenal and neurological tissue to name a few. Other cells types that may be used

in accordance with the present invention are immune cells (such as T-cells, B-cells, natural killer cells, etc.), macrophages/monocytes, adipocytes, pericytes, fibroblasts, neuronal cells, reticular cells etc. In a further embodiment, secondary cell lines may be used, including, but not limited to hepatic cell lines, such as CWSV, NR, Chang liver cells, or other cell lines such as CHO, VERO, BHK, Hela, COS, MDCK, 293, 373, CaSki and W138 cell lines. In a preferred embodiment, host cells comprise one or more cell receptors that facilitate binding or viral infection.

In a preferred embodiment of the invention, the cell is eukaryotic, preferably a cell line, preferably mammalian, and most preferably human may be used for the method of the invention. Cells may be derived from human (*e.g.*, HeLa cells), primate, mouse, rabbit, chicken, etc., although may also be from a transgenic non-human animal. Numerous eukaryotic cell lines may be purchased from ATCC (American Type Culture Collection, Rockville, MD). In a most preferred embodiment, eukaryotic cell is derived from a human. In other embodiments, the cell is derived from a mouse, monkey, or rat. Preferably non-tumor cell lines and autologous permissive cells are used in preparing the FRMS of the subject invention.

Thus, the present invention provides cells comprising one or more components which result in the formation of an FRMS of the invention. The present invention also provides cells comprising all of the components required for the formation of the FRMS of the invention. In one embodiment, the present invention provides a cell that recombinantly expresses an envelope protein of an enveloped virus that functions in fusion of the viral envelope with a host cell membrane, or a mutant form of said envelope protein that is fusion-defective. In a further embodiment the cell expresses one or more cellular membrane proteins that function as receptors for said envelope protein. In a specific embodiment, the invention provides a cell that recombinantly expresses HIV gp160, human CD4 and a co-receptor for HIV.

The present invention also provides a cell line that recombinantly expresses an envelope protein of an enveloped virus that functions in fusion of the viral envelope with a host cell membrane, or a mutant form of said envelope protein that is fusion-defective. In a further embodiment the cell line expresses one or more cellular membrane proteins that function as receptors for said envelope protein. In a specific embodiment, the invention provides a cell line that recombinantly expresses HIV gp160, which cell line expresses CD4 and a co-receptor for HIV; said cell line lacking a functional protease that cleaves gp160 to produce gp120 and gp41.

In other embodiments of the invention, cells comprising the FRMS of the invention are administered as an immunogen(s) to a subject (see Section 5.5, herein). In a

preferred embodiment, when the subject is a human, the cells are human primary cells. In a further preferred embodiment, the cells are autologous.

5.1.4. USING FUSION DEFECTIVE MUTANTS IN FORMATION OF THE COMPLEX

In one embodiment, any viral envelope protein containing wild type fusion activity is used to make the FRMS. In an alternative embodiment, a fusion-defective envelope protein is used, as described in this section.

The present invention provides for the use of mutations to viral envelope proteins or host cell receptor or co-receptor proteins which mutations inhibit the completion of process of viral envelope and cell membrane fusion. The invention provides fusion-defective mutations which do not allow completion of the process of viral envelope-cell membrane fusion, but which do result in the formation of a fusion-related molecular structure of the invention. The fusion-defective mutations are used to construct epitopes comprising an FRMS. In one embodiment of the invention, association of the fusion defective envelope protein with the cellular receptor(s) results in the accumulation of the FRMS. Thus the fusion-defective mutations of the invention may be used in the formation of an FRMS.

Any technique for mutagenesis known in the art can be used to modify individual nucleotides in a DNA sequence, for purpose of making amino acid substitution(s) in the expressed peptide sequence, for creating/deleting restriction sites, or for adding affinity tags. Such techniques include but are not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), oligonucleotide-directed mutagenesis (Smith, 1985, Ann. Rev. Genet. 19:423-463; Hill et al., 1987, Methods Enzymol. 155:558-568), PCR-based overlap extension (Ho et al., 1989, Gene 77:51-59), PCR-based megaprimer mutagenesis (Sarkar et al., 1990, Biotechniques, 8:404-407), etc. Modifications can be confirmed by DNA sequencing.

A fusion-defective mutation may be constructed for any enveloped virus. In a specific embodiment, mutations are constructed that affect the ability to complete fusion. Selection of mutations may be based on published work, or based on molecular modeling from known or hypothetical structure predictions. Mutations known not to detrimentally affect envelope protein expression and transit to the cell surface will be preferred.

In a further embodiment, a fusion-defective mutant is confirmed by expressing the mutated gene in a cell (*e.g.*, the methods of the invention) such as COS, 293T and assayed for fusion competence. Cells may then be assayed for cell surface expression of the mutant component (by methods known in the art such as, *e.g.*, by flow cytometry or by live cell indirect immunofluorescence). Additionally, proteolytic

processing of a mutant protein such as a mutant of HIV gp160 may be assayed by Western blot analysis or any other methods known in the art. Fusion competency of mutant components may be assayed as described in Section 5.7, herein, (*e.g.*, by syncytium formation assay, *e.g.*, in the case of HIV, U87-CD4-co-receptor cell fusion assay).

In a further embodiment of the invention, binding-defective, fusion-defective or fusion-arrested mutant envelope proteins are tested for the ability to elicit primary isolate virus neutralizing antibodies in the transgenic mouse vaccination assay. In this embodiment, immunogens may comprise cells expressing the test mutant envelope protein cocultured with *e.g.* U87-CD4-CCR5 cells (when testing HIV related mutants).

In a preferred embodiment of the invention, mutations that elicit broad PI virus neutralization are developed as subunit and recombinant virus-based immunogens. Table I provides a list of exemplary mutations which are provided for HIV. As will be apparent to one skilled in the art, similar mutations can be constructed for other enveloped viruses.

TABLE I:

Protein/site	Mutation(s)
gp120-gp41 cleavage site	gp120 C terminus REKR to REKT
fusion peptide mutations	gp41 V2E, G10V
gp41 coiled-coil mutations	envelope V570R, Y586E, L568A, W571R, Q577R, N656L
enhanced gp120 cleavage	alteration in furin protease cleavage site of gp120:C-terminus REKR to RSKR

Additional mutations within *e.g.* the bridging sheet of the gp120 core structure include those of Kwong et al. (Kwong PD, et al., 1998, Nature 393:648-59; Kwong PD, et al., 1999, J Biol Chem. 274:4115-23) and regions of putative gp41/gp120 interaction.

In another embodiment, the FRMS of the subject invention are prepared using viral envelope proteins containing binding and fusion-related mutations. In one embodiment, the viral proteins bind host cell receptors, however, mutation causes the fusion process to be arrested before or during fusion of the viral envelope and host cell membrane. Thus, the FRMS of the subject invention include epitopes resulting from the association of one or more viral envelope proteins and one or more cell membrane proteins, including those proteins which are fusion-defective, fusion-arrested or binding-defective so long as

the mutant proteins when used in the methods of the invention result in an epitope elicits PI neutralizing antibodies. Protein complexes arrested during the process of fusion can be isolated and used in the methods and compositions of the invention including but not limited to immunogens, diagnostics, kits, vaccines and compositions.

For example, in a specific embodiment, the FRMS comprise the major viral envelope protein of HIV-1. There are a variety of classes of fusion-related mutations which may be used in constructing the complexes of the subject invention. Without limitation as to mechanism, during HIV-1 and host cell fusion, the oligomeric envelope protein complex gp160, comprising the gp120 surface and gp41 transmembrane portions, binds initially to the CD4 receptor on the host cell. Conformational changes in both the envelope protein and CD4 receptor facilitate interaction with a co-receptor molecule on the host cell surface. Further conformational changes in the viral protein-CD4-co-receptor trimolecular complex allow exposure of a viral gp41 pre-hairpin intermediate and the insertion of the N-terminal gp41 hydrophobic fusion domain into the host cell membrane. Helical heptad-repeat regions within the pre-hairpin intermediate subsequently collapse to form the trimeric, coiled-coil core of fusion active gp41. The coiled-coil core drives the ultimate fusion of the apposed cell and virus membranes.

Viral envelope proteins with mutations that abrogate proteolytic cleavage of the gp160 precursor protein preventing liberation of the gp41 hydrophobic fusion domain can be used to construct the subject complexes. In particular, it has been shown that certain mutations that alter the highly conserved lys/arg-X-lys/arg-arg site at the C-terminus of gp120 abrogates fusogenicity (see e.g., Lee CN, et al., 1994, AIDS Res. Hum. Retroviruses.10:1065-9).

Additionally, mutations that affect the N-terminal gp41 fusion peptide can be used in creating the complexes of the subject invention. The hydrophobic N-terminal region of gp41 mediates fusion by inserting into the cell membrane and destabilizing the lipid bilayer, perhaps by adopting a helical structure. Certain amino acid changes within this region render the envelope protein nonfusogenic. Synthetic peptides bearing these changes are either unable to bind to lipid bilayers unable to elicit fusion. Two fusion-defective mutations in gp41 have been well characterized: V2E and G10V (Kliger Y, et al., 1997, J Biol Chem.272:13496-505). The former involves a polar substitution in the hydrophobic peptide, whereas the later may affect the helical structure assumed in the lipid bilayer. These mutations can be introduced into the 168P envelope gene (gp41: ala-val-gly-ile-gly-val-leu-phe-leu-gly-phe-leu-gly...) by site-directed mutagenesis or any method known in the art and the envelope proteins tested for expression and fusogenicity.

Further, envelope proteins having mutations that alter the coiled-coil core region of gp41 can be used to construct the structures of the subject invention. The

highly-conserved coiled-coil motif is found in proteins involved in fusion of many virus families, and similar structures have been identified in cellular proteins that mediate vesicular fusion. Synthetic peptides that mimic either the N– or C-helical region (*e.g.* DP107 and DP178) (Furuta et al., 1998, Nature Structural Biology 5:276-279.) potently neutralize virus infectivity, presumably by binding the cognate helical region and interfering with the collapses of the pre-hairpin intermediate. In addition to the detailed structural information available from crystallography, the N– and C-helical regions of gp41 have also been saturated by mutagenesis. Several mutations have been identified that retain normal envelope protein expression and assembly yet are non-fusogenic, presumably though disruption of the interface between N– and C-helical coils (*e.g.* V570R and Y586E) (Weng et al., 1998, Journal of Virology 72:9676-9682).

Competent or mutated viral envelope protein as well as cell receptors and/or co-receptors can be incorporated into viral vectors or diploid cells by methods known in the art including those described herein. Viral genes encoding viral proteins are routinely cloned for expression in bacterial cells, in eucaryotic or procaryotic cells, or in viral or DNA vectors. Likewise, human cellular receptors can be cloned and expressed to produce models by which to study dangerous pathogenic diseases. Viral genes or host receptors can be incorporated into foreign DNA by transfection or through the use of viral vectors.

5.1.5. OTHER METHODS TO FORM THE FRMS

Further, other methods by which to trigger formation of the subject structures are contemplated including manipulation of pH conditions, temperature and salt concentration. Additionally, monoclonal antibodies that bind strongly enough to envelope protein may trigger the conformational changes necessary to form the subject structures. Thus, the FRMS of the subject invention can be formed as a result of the interaction of viral proteins and a variety of animal cellular components including but not limited by those listed above.

5.2. CAPTURING THE FRMS

Once a FRMS is formed a variety of methods may be used to preserve or "capture" the FRMS in an immunogenic form that provides vaccine efficacy for primary viral isolates.

In one embodiment, the FRMS of the subject invention result from interaction of one or more viral envelope proteins with one or more host cellular receptors and/or co-receptors. In one embodiment, complexes are "captured" by fixing or cross-linking the FRMS. For example, a FRMS formed in co-cultured cells expressing the viral

and cellular components respectively, which result in a FRMA may be captured by fixation of the cells with a cross-linking agent.

In one specific embodiment, cells transformed with a nucleic acid expressing a viral envelope protein and cells transformed with nucleic acids expressing host cellular receptors and co-receptors are cultured together. The viral protein expressed on the surface of a first cell binds to the receptor(s) expressed on the surface of a second cell. The cells are fixed at the initiation of this cell-cell interaction. Without limitation as to mechanism, crosslinking "captures" the intermediate fusion complex and provides fusion-related determinants which may be isolated and use as, for example, vaccine components. Any method known in the art may be used to express the viral or cellular components of a fusion complex in a cell (See, for example, Section 5.1.1, herein).

In capturing the FRMS, fusing cells or fusion-defective mutant cells can be fixed or cross-linked by any method known in the art. Cross-linking reagents that can be used include but are not limited to formaldehyde glutaraldehyde, formalin, p-Azidobenzoyl hydrazide, N-(4-[p-Azidosalicyclamido]-butyl)-3'(2'-pyridyldithio)-propionamide, Bis(beta-[4-azidosalicylamido]-ethyl)disulfide, 1,4-bismaleimidyl-2,3-dihydroxybutane, 1,6-Bismaleimidohexane, 1,5-Difluoro-2,4-dinitrobenzene, Dimethyl adipimidate-2HCl, Dimethyl suberimidate-2HCl, Dimethyladipodimidate-2HCl, Dimethyl pimelimidate-2HCl, Disuccinimidyl glutarate, Disuccinimidyl tartrate, 1-Ethyl-3-[3-Dimethylanonopropyl] Carbodiimide Hydrochloride, (N-Hydroxy succinimidyl)-4-Azidosalicylic acid, Sulfosuccinimdyl 2-[7-azido-4-methyl-coumarin-3-acetamidomethyl-1,3-aminopropionate, N-Succinimidyl-4-iodoacetylaminobenzoate, N-Succinimidyl-3-[2-pyridylthio]propionate, and Succinimidyl 6-[3-(2-pyridylathio)-propionamide] hexanoate (Pierce Chemical Co., Rockford, IL).

In a preferred embodiment of the invention, the cross-linking reagent is formaldehyde. In one specific embodiment low levels of formaldehyde (0.2%) are used in order to conserve antigenicity. In other embodiments, higher concentrations however can be used to optimize stability. Formaldehyde in concentrations of from about 0.01% to about 8% can be used in preparing the complexes of the subject invention. Other agents that can be used to cross-link fusing cells include but are not limited to glutaraldehyde (0.05-0.5%). One skilled in the art having the benefit of the disclosure contained herein, will recognize that fixing or cross-linking conditions (*i.e.*, reagent used, concentration of reagents, time and temperature) can be varied to determine optimal conditions for immunogenicity of the FRMS.

In a further embodiment, the complexes of the subject invention can be prepared by infecting human diploid cells in cell culture with, for example, recombinant vaccinia virus expressing viral envelope proteins and CD4/co-receptor (if not endogenously

present). During fusion, the cells are cross-linked to capture the fusion-related structures and determinants.

In yet another embodiment of the invention, when cells expressing the cell receptor(s) and/or co-receptors for a virus are infected directly with the live or attenuated virus, the cells undergoing infection may be cross-linked in order to capture the FRMS.

In other embodiments of the invention, a single cell may be engineered to express all of the components important for the formation of a FRMS. In this embodiment, such a cells or cells may be cross-linked or fixed in order to capture the FRMS. In this embodiment, it is not necessary that a cell-cell interaction occur at the time of fixation, since all components are expressed on an individual cell.

The invention provides viral particles which are engineered to express in the viral envelope the cellular components or proteins important for FRMS formation. In this embodiment, the recombinant viral particles may be cross-linked to capture the FRMS formed withing the viral particle envelope.

In an alternate embodiment of the invention, the complexes need not be fixed or cross-linked. It has been found that the complexes of the subject invention form in cell lysates, in solution. Therefore, creation of the subject complexes by, for example, the simple lysis of cells transfected or infected with HIV-1 envelope protein as well as CD4/co-receptor where the FRMS form in solution. Cell lysis protocols are well known in the art (see, *e.g.*, Sambrook et al., *supra*). Cell lysis protocols may involve detergent lysis (*e.g.*, 1% NP40) freeze thaw lysis, sonication lysis, or any method known in the art. In a specific embodiment, cell cultures are fixed at the onset of cell-cell interactions by fixation in ice cold 0.2% formaldehyde in phosphate buffered saline. In another embodiment of the invention detergent lysis of cells involves the use of the detergent Brij 97.

Accordingly, the invention provides a molecular structure which is a cross-linked cellular molecular structure or is isolated from a cell lysate. In other embodiments of the invention provides fusion-defective mutations which allow accumulation of the FRMS and allows capturing of the FRMS.

5.3. ISOLATING THE FRMS

In one embodiment, cells (e.g. cross-linked cells) expressing the components whose association results in the FRMS, can be used as immunogen in the vaccines of the invention. Alternatively, molecular structures comprising the FRMS may be isolated for use as immunogen.

Once the FRMS is formed, it may be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique

for the purification of proteins. In addition, the components important in the formation of a FRMS can be synthesized comprising an affinity tag which facilitates recovery and purification. The peptide tag can be associated with any portion of the protein, so long as such association does not alter the epitope formation generated by association of the modified component with other members of the complex. In various embodiments, such a chimeric protein comprising an affinity tag can be made by ligating a gene sequence coding for the component to the sequence encoding the peptide tag in the proper reading frame and recombinantly expressing the protein. Care should be taken to ensure that the modified gene remains within the same translational reading frame, uninterrupted by translational stop signals.

A variety of peptide tags known in the art may be used in the modification of a protein, such as but not limited to the polyhistidine sequence (Petty, 1996, Metal-chelate affinity chromatography, in Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience), glutathione S-transferase (GST; Smith, 1993, Methods Mol. Cell Bio. 4:220-229), the *E. coli* maltose binding protein (Guan et al., 1987, Gene 67:21-30), and various cellulose binding domains (U.S. Patent Nos. 5,496,934; 5,202,247; 5,137,819; Tomme et al., 1994, Protein Eng. 7:117-123), S TAG™ System (Novagen, Inc.), etc. Other possible peptide tags are short amino acid sequences to which monoclonal antibodies are available, such as but not limited to the following well known examples, the FLAG epitope, the myc epitope at amino acids 408-439, the influenza virus hemagglutinin (HA) epitope. Other peptide tags are recognized by specific binding partners and thus facilitate isolation by affinity binding to the binding partner, which is preferably immobilized and/or on a solid phase surface. As will be appreciated by those skilled in the art, many methods can be used to obtain the coding region of the above-mentioned peptide tags, including but not limited to, DNA cloning, DNA amplification, and synthetic methods. Some of the peptide tags and reagents for their detection and isolation are available commercially.

DNA sequences encoding desired peptide tags which are known or readily available from libraries or commercial suppliers are suitable in the practice of this invention. These and other peptide tags are recognized by specific binding partners and thus facilitate isolation by affinity binding to the binding partner which can be immobilized onto a solid phase surface. The chimeric protein gene product can be prepared using recombinant DNA techniques. For example, gene sequence encoding a component important in the formation of a FRMS can be introduced into a vector containing the sequence of a peptide tag, such that the component gene is expressed as a peptide-tagged chimeric protein. Peptide tags, which may be recognized by specific binding partners, may be used for affinity binding to the binding partner immobilized on a solid phase surface.

In one preferred embodiment, a poly-histidine tagged fusion-related protein is constructed by insertion of a fusion-related protein gene or gene fragment into an expression vector such as one of the pET15 series of vectors (Novagen), that express inserted sequences as chimeric proteins with N-terminal poly-histidine tags. Proteins which have a succession of six or more histidine residues at their amino or carboxyl terminus have a strong binding affinity to nickel. Poly-histidine-tagged chimeric proteins will bind specifically to the surface of a solid phase coated with chelated nickel. In a specific preferred embodiment, microtiter plates coated with metal chelates are used to isolate the poly-histidine-tagged chimeric proteins (Pierce).

Alternatively, for example, a system described by Janknecht, et al. allows for the ready purification of non-denatured chimeric proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88, 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

In another specific embodiment, an expression construct can be made by subcloning a fusion-related protein into the EcoRI restriction site of each of the three pGEX vectors (glutathione S-transferase expression vectors; Smith et al., 1988, Gene 7:31-40). This allows for the expression of a chimeric protein comprising the fusion-related protein linked to the GST binding domain in all three reading frames, such that, in one frame, the GST binding activity is maintained in the resulting chimeric protein. A GST chimeric peptide has a strong binding affinity for its substrate, glutathione. In a specific embodiment, FRMS comprising a GST tag is separated from a binding mixture comprising a cellular lysate or fixed cells by contacting such mixture with a glutathione-linked solid phase surface, such as glutathione sepharose beads. For example, the GST-chimeric protein can be anchored to glutathione-agarose beads or a glutathione-sepharose column. The mixture is then added to the column or beads in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away. The interaction between the chimeric protein and the glutathione-agarose beads allows isolation of the complex.

In another embodiment, a chimeric protein may be readily purified by utilizing an antibody specific for the chimeric protein being expressed.

In yet another embodiment, an affinity-tagged FRMS can be constructed by conjugation of an affinity compound to the fusion-related protein. Affinity compounds can be used, such as, but not limited to, biotin, photobiotin, or other compounds known in the

art. In one embodiment, affinity compounds or affinity tags can be conjugated to the fusion-related protein through a polyfunctional crosslinker, and preferably a bifunctional molecule. As used herein, the term polyfunctional crosslinker encompasses molecules having more than one functional group that reacts with a functional group on the fusion-related protein. Typically, such crosslinker forms covalent bonds with an amino or sulfhydryl group on a polypeptide. For example, biotin N-hydroxysuccinimide esters may be used.

In a preferred embodiment of the invention, a highly-specific method to isolate the FRMS via association with CD4, of a chemok co-receptor, or with envelope protein is the use of S-peptide affinity tagged molecules that takes advantage of the specific and high affinity interaction (K_d=10^{-9} M) of the 15 amino acid S- peptide of RNase A and the larger subtilisin-digest fragment designated RNase S (S-protein; amino acids 21-124 of RNase A) (Potts et al., 1963; Dorai et al., 1994). This S·TAG™ system was developed commercially by Novagen, Inc. for use in the detection and isolation of recombinant chimeric proteins expressed in bacterial and baculovirus systems, and has recently been extended to mammalian chimeric proteins. The fusion-related proteins of the FRMS is tagged with S-peptides. The invention provides examples of components of complexes which were tagged with the S-peptide at the C-terminal ends of the CD4, CCR5 or envelope protein molecules. Tagged complexes were readily isolated and purified by S-protein agarose affinity chromatography.

In one embodiment membrane-impermeable cross-linking reagents can be used to fix cells containing tagged complexes. In one specific embodiment, fixing cells containing tagged complexes with a membrane impermeable cross-linking agent facilitates avoiding inactivation of the cytoplasmic S-peptide tag. Alternatively, the S-peptide sequence may be altered (ala-glu-thr-ala-ala-ala-ala- phe-glu-arg-gln-his-met-asp-ser) so that it no longer contains vulnerable lysine residues and thus can be fixed with formaldehyde without resulting in inactivation of the cytoplasmic tag. Fusing cells can be fixed with a wide range of membrane impermeable cross-linking reagents including, but not limited to BS^3 and DTSSP (homobifunctional N-hydroxysuccinimidyl (NHS) esters that react through amines to form either non-cleavable or reversible linkages, respectively); Sulfo-SMPB (heterobifunctional NHS ester and maleimide to irreversibly cross-link amine and sulfhydryl groups); Sulfo-SANPAH and SASD (heterobifunctional NHS ester and photoreactive phenylazide cross-linkers which form either non-cleavable or reversible linkages, respectively) (Pierce Chemical Company).

Tagged complexes are readily purified providing an isolated preparation of the fusion-related components which may be used for example, for inclusion in vaccine formulations. Purified or isolated tagged complexes can also be used as diagnostic

standards for *in vitro* assays. Further, tagged complexes permit the functional analysis of envelope-mediated fusion.

Accordingly, the invention provides a method of purifying a protein complex comprising an envelope protein of human immunodeficiency virus type 1 functionally interacting with human CD4 and human CCR5 including the steps of: a) tagging the complex with a peptide sequence to facilitate subsequent purification; and b) isolating the tagged complex.

In other specific embodiments, the invention provides an isolated protein complex comprising an envelope protein of human immunodeficiency virus type 1 functionally interacting with human CD4 and human CCR5.

5.4. TYPES OF FRMS

As will be apparent to one skilled in the art, any enveloped virus may be used in the methods of the invention in order to construct or use the FRMS. For example, an important use of the FRMS of the invention is as vaccine immunogens. One advantage of the vaccines comprising FRMS is that these immunogens provide significantly higher neutralization rates of primary isolates. The enveloped viruses that can be used include but are not limited to the families of Retroviridae, Rhabdoviridae, Coronaviridae, Filoviridae, Arenaviridae, Poxviridae, Bunyaviridae, Flaviviridae, Togaviridae, Orthomyxoviridae, Paramyxoviridae, Herpesviridae, and Iridoviridae.

In one embodiment of the invention, a retroviral FRMS is formed by the methods of the invention. For example, in a preferred embodiment the FRMS of the invention comprises the major viral envelope immunogen of human immunodeficiency virus type 1 (HIV-1) and arises from interaction with the host cellular receptor for HIV-1 and a host cellular co-receptor. The host cellular co-receptor can be CCR5, CXCR4, CCR3, CCR2b or any other known in the art. An exemplified FRMS of the present invention results from interaction of the HIV-1 isolate 168P viral envelope protein arising from interaction with the host cellular receptor CD4, and the host cellular co-receptor CCR5. It should be apparent to those skilled in the art that other recombinant HIV envelope proteins, including those that act independently of CD4, could be used to prepare FRMS within the scope of the subject invention.

In a specific embodiment, by way of example but not limitation, for an HIV vaccine, the immunogens of the subject invention are prepared by co-culturing envelope-protein expressing COS-7 cells (LaCasse et al. 1998, Science, 283:357-360) and cells expressing the human CD4 receptor and CCR5 co-receptor (U87-CD4-CCR5). In this embodiment, the envelope-expressing cells are harvested using 0.5 mM EDTA 24 hr after transfection (30-60% transfection efficiency) and co-cultured with an equal number of

U87-CD4-CCR5 target cells. Progress towards fusion is assessed by staining envelope-expressing cells and monitoring their incorporation into multinucleated syncytia microscopically. Fusion is complete within 12-24 hr. Complexes are formed immediately. Cells fixed after 1-5 hrs of co-culture are at an estimated 10-30% of maximal syncytium formation. This early time point in fusion was chosen initially in order to capture the transitional intermediates that lead to fusion. Other time points (*e.g.* 10 hr, 24 hr or greater) can also be used to obtain the fusion-related complexes of the subject invention. Complexes from later points in the fusion process may reveal still further neutralizing epitopes and can be used to map appearance and course in time of critical immunogens.

In other embodiments, Flavivirus FRMS is generated by the methods of the invention. Viruses within the Flavivirus family include, for example, Hepatitis C Virus (HCV), Dengue virus, Yellow Fever virus, Tick-borne Encephalitis virus, and Bovine Viral Diarrhea virus. As a family, the Flaviviruses are similar to the Togaviridae which include the alphaviruses (*e.g.*, Venezuelan Equine Encephalitis virus, Sindbis virus, Semliki Forest virus) and Rubella virus. By contrast to HIV, the Flaviviruses enter target cells via receptor-mediated endocytosis followed by pH-induced fusion within the endosome.

Viruses within the Flavivirus family encode an envelope (E) protein which is proteolytically cleaved in many family members to mature E1 and E2 proteins. In the specific case of HCV (Hepatitis C Virus), the E2 protein is the receptor binding moiety (binding the cellular membrane protein CD81) and E1 is the putative viral fusion protein.

In another embodiment of the invention, a Flavivirus or Togavirus FRMS is formed by the methods of the invention. For example, an HCV FRMS of the invention results from the association of E1, E2 and CD81. In a specific embodiment, the HCV E1 and E2 proteins can be co-expressed in an acceptable cell substrate and will become localized in the cell endoplasmic reticulum (ER) and not significantly on the cell surface. In one embodiment, these cell cultures are incubated in medium buffered to pH 5 to 6.8 in order to trigger E1E2 fusion of the intracellular membrane. The cultures are treated with a cross-linking agent to capture the intermediate fusion-competent structures, and used directly (or upon isolation of intracellular ER membranes) as vaccine immunogen.

In another specific embodiment, the disrupted intracellular ER membranes containing E1E2 protein are isolated as everted micelles and then incubated with an appropriate permissive cell expressing the E2 receptor, CD81. E1E2-containing micelles are internalized and E1-mediated fusion occurs in the endosome. Cells are then cross-linked and used for example, as vaccine immunogens. Alternatively, E1E2 proteins are engineered to alter ER retention signals and thus allow expression of E1E2 proteins on the cell surface thus obviating the need to isolate ER membranes.

In another specific embodiment, recombinant DNA derived HCV virus-like particles or HCV E1E2 containing pseudotyped virions (*e.g.* an alphavirus or VSV) can be produced and triggered for fusion by incubation with receptor-expressing cells in medium adjusted to pH 5-6.8. The cultures are treated with a cross-linking agent such as formaldehyde and the FRMS is used as a vaccine immunogen. Alternatively, virions can be incubated with CD81-expressing cells; internalized virions will be induced to fuse within the endosome and can be captured by cross-linking treatment for use as a vaccine immunogen of the invention.

In yet another embodiment of the invention, an orthomyxovirus FRMS is generated by the methods of the invention. The orthomyxoviridae family includes all influenza viruses, including but not limited to, influenza A, influenza B and influenza C. Thus, in a preferred embodiment, an influenza FRMS elicits neutralizing antibodies against a wide variety of flu strains and would allow for a drift-independent flu vaccine.

Like the Flaviviruses and Togaviruses, the Orthomyxoviruses enter target cells via receptor-mediated endocytosis and fusion within the endosome. In this case, the viral neuraminidase (NA) protein functions as the sialic acid receptor binding protein (a ubiquitously expressed cell surface carbohydrate), and the hemagglutinin (HA) protein functions in pH-induced fusion. In contrast to the Flaviviruses, Influenza virus buds from the cell surface and thus recombinant NA and HA protein expression occurs on the cell surface.

In other embodiments influenza FRMS is generated by the methods of the invention. Influenza FRMS is generated by the methods similar to those described above for the Flaviviruses and include pH-triggered HA-mediated fusion between appropriate cells or between cells and isolated cell membranes or virion particles, capturing the complex may be performed, for example, with a cross-linking agent allowing NA-mediated endocytosis and subsequent endosomal fusion to be captured.

In another embodiment of the invention, a paramyxovirus FRMS is generated by the methods of the invention. The Paramyxovirus family encompasses several subgroups of significant medical and veterinary importance, including but not limited to, Morbilliviruses (measles virus, Canine Distemper virus, Rinderpest virus), Rubulaviruses (Mumps virus, Newcastle Disease), paramyxoviruses (human and bovine parainfluenza), and Pneumoviruses (Respiratory Syncytium virus). These viruses are similar to the Orthomyxoviruses, except that Paramyxovirus fusion occurs at the cell surface rather than in the endosome, and is pH independent. For the Paramyxoviruses, the neuraminidase-hemagglutinin (HN) (or simply hemagglutinin (H)) protein mediates cell attachment whereas the fusion (F) protein mediates virus-cell or cell-cell fusion. For example, in the case of measles virus, the H protein binds the cellular CD46 receptor and the F protein

mediates membrane fusion. In a specific embodiment, cells expressing H and F proteins from MV are co-cultured with cells expressing CD46 (and currently unidentified co-receptors) and result in cell-cell fusion that can be captured by cross-linking or other methods described herein. Alternatively, in another embodiment the co-receptor may be expressed on fusion-permissive cells expressing exogenus CD46. Specific embodiments (as described for HIV and other viruses above) may also be used.

In one embodiment of the invention, a herpesvirus FRMS is generated by the methods of the invention. The Herpesviruses comprise a large family that include members of significant medical and veterinary importance. The human herpesviruses include, but are not limited to, HSV 1 and HSV 2, Varicella-zoster virus, Epstein-Barr virus, Cytomegalovirus, and Kaposi's Herpesvirus HHV 8.

For example, in the case of HSV 1, binding and entry is a complex process involving several viral glycoproteins (gD, gC, and gH/gL) and several cell receptors and adhesion molecules (glycosaminoglycans and Herpesvirus entry mediator (Hve) proteins A-C). Binding and fusion occur on the cell surface in a pH-independent manner.

In a specific embodiment, HSV FRMS is constructed using a cell expressing the cellular receptors and adhesion molecules (*e.g.* human MRC5 cells) and co-culturing such cells with cells expressing the HSV glycoproteins gD, gC, gH, and gL. Cell-cell fusion is preferably arrested by fixation with a cross-linking agent, to allow capturing of the FRMS.

As described herein, viral proteins and host cellular receptors can be combined to form the subject FRMS for viruses including Herpes virus, pox viruses, paramyxovirus, measles, mumps, rubella, respiratory syncytial virus, influenza, Hepatitis C, ebola and flaviviruses such as Dengue and Yellow Fever. Additionally, the FRMS of the subject invention can include viral proteins from viruses of veterinary importance including rabies, feline leukemia, feline immunodeficiency virus and rinderpest.

Table II presents an exemplary list of the cellular receptor(s) for particular enveloped viruses, which when expressed along with the viral envelope protein and allowed to associate with the envelope protein results in FRMS formation.

TABLE II:

Virus	**Cellular Receptor(s)**
Bovine coronavirus	N-acetyl-9-O-acetylneuraminic acid receptor
Choriomeningitis virus	$CD4^+$

Virus	Cellular Receptor(s)
Dengue virus	Highly sulphated type heparin sulphate p65
Ebola	CD16b
Feline leukemia virus	Extracellular envelope glycoprotein (Env-SU) receptor
Gibbon ape leukemia virus (GALV)	GALV receptor
Herpes Simplex Virus	Heparin sulphate glycosaminoglycan receptor Fibroblast growth factor receptor
HIV-1	CD4 CC-Chemokine receptor CCR5 Chemokine receptor CXCR4
Human cytomegalovirus	Heparin sulphate proteoglycan Annexin II CD13 (aminopeptidase N)
Human coronovirus	Human aminopeptidase N receptor
Influenza A, B & C	Hemagglutinin receptor
Measles virus	CD46 receptor
Morbilliviruses	CD46 receptor
Mouse hepatitis virus	Carcinoembryonic antigen family receptors Carcinoembryonic antigen family Bg1a receptor
Murine leukemia virus	Envelope glycoproteins
Murine gamma herpes virus	gamma interferon receptor
Murine retrovirus	Glycoprotein gp70 Rmc-1 receptor
Murine coronavirus mouse hepatitis virus	Carcinoembryonic antigen family receptors
Newcastle disease virus	Hemagglutinin-neuraminidase protein Fusion protein
Pox Virus	Interferon gamma receptor
T-cell lymphotropic virus 1	gp46 surface glycoprotein

Virus	Cellular Receptor(s)
Vaccinia virus	TNFRp55 receptor TNFRp75 receptor Soluble Interleukin-1 beta receptor

Other viral diseases that can be treated or prevented by the methods of the present invention include, but are not limited to, those caused by hepatitis type C, influenza, varicella, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, respiratory syncytial virus, cytomegalovirus, echinovirus, arbovirus, hantavirus, mumps virus, measles virus, rubella virus, human immunodeficiency virus type I (HIV-1), human immunodeficiency virus type II (HIV-2), any togaviruses (such as Dengue virus), alphaviruses, flaviviruses, coronaviruses, rabies virus, Marburg viruses, ebola viruses, parainfluenza virus, orthomyxoviruses, bunyaviruses, arenaviruses, human T cell leukemia virus type I, human T cell leukemia virus type II, simian immunodeficiency virus, lentiviruses, Epstein-Barr virus, human herpesvirus-, cercopithecine herpes virus 1 (B virus), and poxviruses.

Table III presents an exemplary list of envelope proteins for particular families of enveloped viruses, which when expressed along with the cellular receptor protein(s) and allowed to associate with the cellular receptor protein(s) results in FRMS formation.

TABLE III:

FAMILY	EXAMPLES OF ENVELOPE PROTEINS
Togaviridae	E2
Flaviridae	E
Coronaviridae	E2 (S)
Rhabdoviridae	G
Paramyxoviridae	HN, H, F
Orthomyxoviridae	HA
Bunyaviridae	G1, G2
Arenaviridae	G1, G2
Retroviridae	gp120, gp41, gp160
Herpesviridae - HSV	gB, gD and gH

- EBV	gp350/220 and gp85
Poxviridae	56-Kd and 14-Kd

In other embodiments of the invention, a triple-stranded coiled-coil structure of Orthomyxoviridae (such as Influenza), Filoviridae (such as Ebola), or Retroviridae (such as HIV) facilitates the formation of FRMS.

In another embodiment, synthetic peptides may be used to inhibit viral infection. For example, by way of illustration, synthetic peptides that comprise either of the gp41 helical coils are able to bind the cognate helical region and broadly inhibit viral infectivity (*see e.g.*, Wild, C., et al., 1992, Proceedings of the National Academy of Sciences USA 89:10537; Furuta, R.A., et al., 1998, Nature Structural Biology 5:276).

In other embodiments, neutralizing antibodies to FRMS immunogens may likewise target structures involved in the activation of fusion.

In another embodiment of the invention, viral strains may be used which do not require a host cell receptor, but require only a host cell co-receptor for association with the virus. For example, in the case of HIV, an HIV strain which does not require CD4 for viral binding may be used in the methods of the invention (*see e.g.*, Hoxie, et al., 1998, J. Reprod. Immunol. 41:197-211.). Therefore, when using such a strain, expression of CD4 is not necessary.

5.5. VACCINE FORMULATIONS AND ADMINISTRATION

The subject invention also concerns methods for vaccinating an individual or animal to raise an immune response to and prevent infection by a virus. The FRMS of the subject invention can be presented to the vaccinee by several means. The FRMS described above can be administered to a vaccinee as fixed cells combined with an adjuvant. Further, isolated and purified FRMS can be administered to a vaccinee.

FRMS can be prepared *in situ* by the simultaneous immunization of a vaccinee with transfected cells expressing viral protein and transfected cells expressing host cellular receptors and/or co-receptors. Likewise, vectors containing DNA encoding viral proteins and vectors containing DNA encoding receptor proteins can be used in this immunization strategy. Vectors can include viral vectors such as vaccinia and vectors used in DNA immunization. After simultaneous immunization, interaction between the viral protein and host cellular receptor protein occurs *in vivo* forming the FRMS of the subject invention and thereby exposing the vaccinee to the unique epitopes capable of eliciting neutralizing antibodies.

Another method by which the FRMS can be formed *in situ* includes utilizing the receptors and co-receptors on host cells. For example, in preparing the exemplified HIV envelope FRMS arising from interaction with CD4 and CCR5, transfected cells expressing the HIV glycoprotein or vectors containing DNA encoding the protein are injected into or targeted (e.g. by VEE or other vectors) the lymph node of a host. The viral protein binds and initiates fusion *in vivo* to host cells having viral receptors and co-receptors to form the FRMS whereby the vaccinee is exposed to the newly formed neutralizing epitopes.

In a specific embodiment, a vaccine formulation comprises an isolated protein complex comprising an envelope protein of human immunodeficiency virus type 1 functionally interacting with human CD4 and human CCR5, and a pharmaceutically acceptable carrier. In another embodiment, the invention provides a method of immunizing an animal to a virus comprising the steps of administering to the animal a vaccine formulation comprising a protein complex comprising one or more viral proteins functionally interacting with one or more host cellular receptors or co-receptors to mediate viral binding, entry and/or infection; whereby neutralizing antibodies to the virus is generated.

In one embodiment, the invention provides a vaccine formulation comprising (a) a first nucleic acid encoding an envelope protein of an enveloped virus; and (b) a second nucleic acid encoding one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins, such that the envelope protein and cellular membrane proteins are expressed in the subject and neutralizing antibodies to the virus are produced; and (c) a pharmaceutically acceptable carrier.

In one embodiment, the invention provides a method of treating a host that has been exposed to a virus, or preventing infection of a host by said virus, the method comprising the steps of administering to the host antibodies generated by immunizing an animal with an isolated protein complex comprising an envelope protein of human immunodeficiency virus type 1 functionally interacting with human CD4 and human CCR5. in an amount effective to treat or prevent infection of said host.

In another embodiment, the invention provides a method of preparing a protein complex comprising one or more viral proteins functionally interacting with one or more host cellular receptors or co-receptors to mediate viral binding, entry and/or infection, including the steps of: a) culturing a first cell expressing one or more viral proteins; b) culturing a second cell expressing one or more host cellular receptors or co-receptors for said one or more viral proteins' c) co-culturing the first and second cells; d) fixing said co-culture during cell-cell fusion; and e) isolating the fixed cells.

Many methods may be used to introduce the vaccine formulations of the invention; these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal routes, and via scarification (scratching through the top layers of skin, *e.g.*, using a bifurcated needle).

The patient or subject to which the vaccine is administered is preferably a mammal, most preferably a human, but can also be a non-human animal including but not limited to cows, horses, sheep, pigs, fowl (*e.g.*, chickens), goats, cats, dogs, hamsters, mice and rats.

The patient or subject to which the vaccine is administered can also be a non-human wild animal including but not limited to lion, cheetah, elephants, giraffe, wildebeest, leopards, panthers, etc. Thus, in this embodiment, the invention provides a means of wildlife management, and provides a method of preventing or treating a wild animal or animal population which has a high degree of inbreeding such that said populations are highly susceptible to viral disease.

In several embodiments of the methods herein, the subject is a human. In other embodiments, said human has a high risk of HIV infection. In other embodiments, the subject is a domestic animal.

The present invention thus provides a method of immunizing an animal, or treating or preventing viral diseases or disorders in an animal, comprising administering to the animal an effective immunizing dose of a vaccine of the present invention.

The vaccine formulations of the present invention can also be used to produce antibodies for use in passive immunotherapy, in which short-term protection of a host is achieved by the administration of pre-formed antibody directed against a heterologous organism.

Vaccination of mice with immunogens comprising FRMS of the subject invention elicits the production of neutralizing antibodies by immunized animals. In an exemplified embodiment, the complexes are administered as a vaccine comprising fixed whole cells formulated with an adjuvant. It would be apparent to one skilled in the art however that other vaccine strategies can be used in practicing the subject invention. For example, isolated and purified FRMS and/or epitopes thereof can be administered as subunit vaccines. Additionally, as mentioned previously, genes encoding the functional complexes can be constructed and placed in vectors or plasmids for use in live vector or DNA plasmid vaccines.

The virus vaccine formulations of the invention comprise an effective immunizing amount of one or more FRMS as a vaccine immunogens of the invention and a pharmaceutically acceptable carrier or excipient. Boosting is also contemplated. In one embodiment, vaccine compositions of the invention can comprise "pharmaceutically

acceptable carriers". As used in this description, pharmaceutically acceptable carriers are substances which do not interfere with the operation of the immunogen and are non-toxic to the animal. Pharmaceutically acceptable carriers are well known in the art and include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, oil-on-water or water-in-oil emulsions, aqueous compositions, liposomes, microbeads, microsomes or adjuvant compounds and combinations thereof. One example of such an acceptable carrier is a physiologically balanced culture medium containing one or more stabilizing agents such as stabilized, hydrolyzed proteins, lactose, etc. The carrier is preferably sterile. The formulation should suit the mode of administration.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is administered by injection, an ampoule of sterile diluent can be provided so that the ingredients may be mixed prior to administration.

In a specific embodiment, a lyophilized fusion-competent complex of the invention is provided in a first container; a second container comprises diluent consisting of an aqueous solution of 50% glycerin, 0.25% phenol, and an antiseptic (*e.g.*, 0.005% brilliant green).

The precise dose of immunogen or FRMS, to be employed in the formulation will also depend on the route of administration, and the nature of the patient, and should be decided according to the judgment of the practitioner and each patient's circumstances according to standard clinical techniques. An effective immunizing amount is that amount sufficient to produce an immune response to the complex in the host to which the fusion-dependent complex, or vaccine immunogen, is administered.

In a specific embodiment, an effective immunizing amount of a vaccine immunogen for a human subject of the present invention is within the range of 10 to100 mg per kilogram body weight, more preferably 0.1 to10mg per kilogram body weight. Boosting is possible but not preferred.

The exact amount of vaccine immunogen utilized in a given preparation is not critical, provided that the minimum amount necessary to provoke an immune response is given. A dosage range of as little as about 10 μg, up to amount a milligram or more, is

contemplated. As one example, in a specific embodiment, individual dosages may range from about 50-650 μg per immunization. In other embodiments, dosing is dependent upon the formulation of the vaccine immunogen (e.g. purified mimitope, vs. purified FRMS, vs. whole cell preparations).

In a preferred embodiment, the vaccine formulation comprises an effective immunizing amount of the FRMS immunogen, preferably in combination with an immunostimulant; and a pharmaceutically acceptable carrier. As used in the present context, "immunostimulant" is intended to encompass any compound or composition which has the ability to enhance the activity of the immune system, whether it be a specific potentiating effect in combination with a specific immunogen, or simply an independent effect upon the activity of one or more elements of the immune response. Some of the more commonly utilized immunostimulant compounds in vaccine compositions are the adjuvants alum or muramyl dipeptide (MDP) and its analogues. Methods of utilizing these materials are known in the art, and it is well within the ability of the skilled artisan to determine an optimum amount of stimulant for a given viral vaccine. It may also be desired to use more than one immunostimulant in a given formulation.

Use of purified immunogens or complexes vaccines can be carried out by standard methods. For example, the purified protein(s) should be adjusted to an appropriate concentration, formulated with any suitable vaccine adjuvant and packaged for use. Suitable adjuvants may include, but are not limited to: mineral gels, *e.g.*, aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols; polyanions; peptides; oil emulsions; alum, and MDP. The immunogen may also be incorporated into liposomes, or conjugated to polysaccharides and/or other polymers for use in a vaccine formulation. In instances where the complex is a hapten, *i.e.*, a molecule that is antigenic in that it can react selectively with cognate antibodies, but not immunogenic in that it cannot elicit an immune response, the hapten may be covalently bound to a carrier or immunogenic molecule; for instance, a large protein such as serum albumin will confer immunogenicity to the hapten coupled to it. The hapten-carrier may be formulated for use as a vaccine.

Effective doses (immunizing amounts) of the vaccines of the invention may also be extrapolated from dose-response curves derived from animal model test systems.

Accordingly, the present invention provides a method of treating or preventing infection by a virus in a subject comprising administering to the subject an immunogenic amount of the FRMS effective to treat or prevent infection by the virus. In a specific embodiment, the present invention provides a method of treating or preventing infection by HIV in a human comprising administering to the human an immunogenic amount of the FRMS effective to treat or prevent infection by HIV.

The present invention also provides a method of treating or preventing infection by a virus in a subject comprising administering to the subject an amount of the FRMS effective to treat or prevent infection by the virus. In a specific embodiment, the invention provides a method of treating or preventing infection by HIV in a human comprising administering to the human an amount of the monoclonal antibody effective to treat or prevent infection by HIV.

5.6. ANTIBODIES PRODUCED

The present invention relates to the formation of polyclonal and monoclonal antibodies reactive to a FRMS of the invention. According to the invention, FRMS, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

The subject invention also concerns methods for generating an immune response, such as antibody production, in an animal. In one embodiment, an animal is administered a FRMS of the invention. Preferably, the FRMS comprises the HIV envelope protein and arises from interaction with CD4 and CCR5. The FRMS is administered in a manner such that an immune response is produced in the animal to the immunogen. Preferably, antibodies to epitopes on the FRMS are produced. More preferably, the antibodies produced include neutralizing antibodies. In a highly preferred embodiment, the antibodies produced are capable of neutralizing a wide variety of primary isolates of the virus.

Various procedures known in the art may be used for the production of polyclonal antibodies to a FRMS or derivative or analog. In a particular embodiment, rabbit polyclonal antibodies to an epitope of FRMS, a subsequence thereof, can be obtained. For the production of antibody, various host animals can be immunized by injection with the native FRMS, or a synthetic version, or derivative (*e.g.*, fragment or chimera) thereof, including but not limited to rabbits, mice, rats, *etc.* Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed to a FRMS, derivative or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique

originally developed by Kohler and Milstein, (Kohler et al., 1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals (*see e.g.*, PCT/US90/022548). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cole et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96).

In a specific embodiment, spleens from mice immunized with FRMS are harvested and splenocytes are isolated by teasing the spleen apart in culture medium. Splenocytes are fused with HPRT-negative mouse myeloma cells using polyethylene glycol (PEG). Cell pellets containing a 4:1 ratio of splenocytes to myeloma cells are resuspended and treated in serum-free medium containing 50% pretested PEG-4000 (2.5 min contact). The cell suspension is then slowly diluted in serum-free medium, and cells are plated in 96-well culture dishes in HAT selection medium: DMEM culture medium containing 20% pretested fetal bovine serum, 10% NCTC-109 (Gibco), 1% nonessential amino acids, 1X glutamine and 1X Pen-Strep, 1X OPI (15 mg/ml oxaloacetate, 5 mg/ml sodium pyruvate, and 20 units/ml insulin), 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine. Wells containing hybridomas are monitored microscopically and supernatants harvested as appropriate/necessary for assay of antibody production.

In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for a FRMS together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. In another embodiment, "humanized" antibodies are also provided by the invention (U.S. Patent No. 5,225,539).

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce FRMS-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab′ expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for tumor suppressor proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited

to, the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule, the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

Fab fragments from combinatorial libraries are also contemplated in the invention (see e.g., Chanock et al., 1993, Infect Agents Dis. 2:118-31).

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (*e.g.*, enzyme-linked immunosorbent assay or ELISA). For example, to select antibodies which specifically recognize FRMS, one may assay generated hybridomas for a product which binds to a FRMS epitope and which does not bind to the epitopes generated by other proteins. For selection of an antibody that specifically binds a first FRMS homolog but which does not specifically bind a different FRMS homolog, one can select on the basis of positive binding to the first FRMS homolog and a lack of binding to the second FRMS homolog. Screening of antibodies may also be performed by functional assays such as virus neutralization assay, such as those known in the art or described herein.

Antibodies specific to a domain of a FRMS is also provided. Antibodies specific to an epitope of a FRMS protein are also provided.

The generated antibodies may be isolated by standard techniques known in the art (*e.g.*, immunoaffinity chromatography, centrifugation, precipitation, etc.) and used in diagnostic immunoassays. The antibodies may also be used to monitor treatment and/or disease progression. Any immunoassay system known in the art, such as those listed *supra*, may be used for this purpose including but not limited to competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme-linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays, to name but a few.

The FRMS of the subject invention, when used as immunogens are capable of eliciting neutralizing antibody to viral pathogens. In a preferred embodiment, the FRMS elicit neutralizing antibody to a wide variety of primary isolates of the virus. In a specific embodiment, the FRMS elicit neutralizing antibody to primary isolates of HIV-1. Neutralizing antibodies, while not elicited by static HIV envelope protein, are able to bind at least weakly to static envelope and are carried with the envelope protein through the binding and fusion process, during which the antibodies complete their high-affinity binding to critical intermediate structures. Such neutralizing antibodies can be, in effect, transported to the critical site to be available at the critical moment regardless of the cellular

site of action, in particular regardless of the cellular site of membrane fusion (either at the cell surface or at internal membranes such as the endosomal membrane). Thus, viral envelope proteins can serve as a vector to bring antibodies (*e.g.* linked to *e.g.* toxin, other protein, vaccine immunogens) into the cell, into the cytoplasm or into the histocompatibility complex presentation pathway.

Hybridoma cell supernatants of the invention can be assayed for the ability to neutralize the homologous virus as well as for the ability to neutralize a range of genetically diverse viruses. The ability to neutralize genetically diverse viruses suggests recognition by the mAb of a conserved determinant. Further, mAb can be tested for inhibition of envelope-mediated cell-cell fusion. MAb can also be assayed for the ability to bind and/or immunoprecipitate envelope protein and envelope protein-containing receptor-associated complexes. Of particular interest are those mAb that are specific for fusion-dependent epitopes and bind only weakly, if at all, to static envelope protein.

In one embodiment, hybridomas are identified using high-throughput screening assay to detect PI virus neutralization, and mAbs are characterized to determine the breadth and molecular targets of PI virus neutralization.

In various embodiments of the invention, the antibodies produced by the methods of the invention are used individually (e.g. a single mAb) or in combination (e.g., one or more mAbs directed to the same or different epitopes). Combinatorial use of antibodies may include multiple mAbs directed to the same virus or to different viruses. In some embodiments of the invention it may be advantageous to use multiple mAbs directed to the same virus or multiple mAbs directed too the same FRMS of a virus.

5.7. ASSAYS FOR ANTIBODY BINDING AND INHIBITION OF VIRAL INFECTION

The ability of antibodies of the invention or the derivatives or analogues thereof to bind FRMS of the invention and thereby interfere with viral infection can be assayed by various methods.

Binding can be assayed by means well-known in the art. For example, bioassays may be performed in which cells known to be expressing a chemokine receptor are exposed to the mAb derivative or analogue to be tested and assayed for a known effect (*e.g.*, signal transduction). Alternatively, mAb, derivatives or analogues can be tested for the ability to bind chemokine receptors, host cell receptors or viral envelope proteins by procedures, including but not limited to, protein affinity chromatography, affinity blotting, immunoprecipitation, cross-linking, and library based methods such as protein probing, phage display, and the two-hybrid system (*see*, generally, Phizicky et al., 1995, Microbiol. Rev. 59:94-123).

High throughput screening for mAb, derivative or analogue binding may be performed by methods known in the art, including but not limited to flow cytometry. In a specific embodiment, cells that express human CD4 and one of the HIV co-receptors (*e.g.*, CC CKR-5, CxC CKR4, CCR5, CXCR4 etc.) are treated with biotinylated mAb, derivative, or analogue and cell surface binding to the FRMS is detected with an avidin FITC conjugate. Alternatively, flow cytometry system may be used in a competitive binding assays using the monoclonal antibodies of the invention in the following manner or by any method known in the art. In a specific embodiment, a mAb of the invention is labeled and examined for the ability to bind an FRMS of the invention compared with the ability of a test antibody to bind to the same FRMS. Test antibodies of the invention may be derived from samples such as serum from a subject, or manufacturing samples such as hybridoma supernatants.

In another embodiment, the anti-viral activity exhibited by the mAb, mAb derivative and/or analogue of the invention may be measured, for example, by easily performed *in vitro* assays, which can test the compound's ability to inhibit syncytia formation or to inhibit infection by cell-free virus and assess the effects of the compound on cell proliferation and viability. Applying these assays, the relative anti-viral activity that a mAb, derivative and/or analogue exhibits against a given virus or strain of immunodeficiency virus formulation best suited for viral and strain specific inhibitory activity can be determined.

In one embodiment, a cell fusion assay is used to test the ability of mAb, derivative or analogue, to inhibit virus-induced syncytia formation *in vitro*. In a specific embodiment, a cell fusion assay is used to test the ability of a mAb derivate or analog of the invention to inhibit HIV-induced syncytia formation *in vitro*. In this embodiment, such an assay involves culturing uninfected $CD4^+$ cells in the presence of chronically HIV-infected cells and the composition containing the mAb, derivative or analogue to be assayed. For each, a range of concentrations may be tested. This range should include a control culture wherein no mAb, derivative and/or analogue has been added. Standard conditions for culturing, well known to those of ordinary skill in the art, are used. After incubation for an appropriate period, such as, for example, 24 hours at 37°C, the culture is examined microscopically for the presence of multinucleated giant cells, which are indicative of cell fusion and syncytia formation.

In another embodiment, an *in vitro* infectivity assay is performed using primary macrophages and the macrophage-tropic isolate HIV-1_{BaL}, the first described macrophage-tropic HIV-1 isolate (*see,* Gartner et al., 1986, Science 233:215). According to this assay, primary macrophage cells isolated according to methods known in the art are infected with HIV-1_{BaL} that has been propagated and maintained only in primary

macrophages. The input immunodeficiency virus is incubated with primary macrophages in the presence of concentrations of the mAb, derivative, or analogue to be tested. After a defined period of infection, unbound virus is removed by washing, and the cells are placed in culture. The level of virus replication in this assay may be assessed by techniques known in the art, including but not limited to, measuring reverse transcriptase (RT) levels, or the release of extracellular p24 core antigen at different days post-infection. A constant level of inhibition of viral infection or replication is determined by measuring output HIV p24 levels (or another indicator of viral infection or replication, such as for example, RT) relative to control assays performed in the absence of the mAb, derivative or analogue. Preferably, the mAb derivative or analogue reduces levels of virus, as measured by, for example, p24, by ≥ 50% relative to control assays carried out in the absence of test compound. The presence of p24 may be determined using methods known in the art, such as commercially available enzyme-linked immunosorbent assays (Coulter, Hialeah, Florida; Abbott Laboratories, Hvalstad, Norway). Alternatively, RT activity may be tested by monitoring cell-free supernatant using standard techniques such as those described by, for example, Goff et al. (Goff et al., 1981, J. Virol. 38:239-248) and Willey et al. (Willey et al., 1988, J. Virol. 62:139-147).

In other embodiments of the invention, a mAb of the invention is assayed in a primary isolate neutralization assay. A variety of neutralization assays are known in the art, and are within the scope of the invention. In one embodiment, mAbs of the invention are assayed in a focus assay. In one embodiment of the focus assay, test antibody is incubated with a measured amount of virus prior to the addition of the virus to host cells. Infection of the host cells is measured by staining said cells by immunohistochemical methods known in the art using an antibody specific for the viral protein(s). Generally, a control assay is performed in parallel in which no test antibody is used. Test antibody which inhibits (e.g., prevents or decreases) infection compared to the control infection is indicative of a neutralizing antibody. In another embodiment of the invention, a PBL assay can be used to assay neutralization by a test antibody. In one embodiment of the PBL assay, test antibody is incubated with a measured amount of virus prior to the addition of the virus to host cells. Host cell cultures are allowed to incubate for a period of days, and infection is assayed by measuring the virions present in a sample of host cell supernatant. Generally, a control assay is performed in parallel in which no test antibody is used. Test antibody which inhibits (e.g., prevents or decreases) infection compared to the control infection is indicative of a neutralizing antibody.

5.7.1. TRANSGENIC MOUSE MODEL

The present invention encompasses the use of a non-human transgenic animal which expresses one or more components whose association results in the formation of the FRMS of the invention. In specific embodiments the components whose association results in the formation of the FRMS may include those listed in Table II and Table III, herein. The transgenic animal models of the invention may be used as Tolerance models and/or Infection models. In the case of Tolerance models, it is preferred that all of the cellular components important to the formation of the FRMS are expressed in the transgenic animal. In the case of Infection models, one or more of the components important for the formation of the FRMS may be expressed such that the transgenic animal is capable of infection by the virus to which the FRMS is derived.

Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, sheep, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate transgenic animals expressing a component which results in the formation of a FRMS. The term "transgenic," as used herein, refers to animals expressing one or more components whose association results in the formation of the FRMS gene sequences from a different species (*e.g.*, mice expressing human sequences encoding one or more component whose association results in the formation of a FRMS), as well as animals that have been genetically engineered to over express endogenous (*i.e.*, same species) sequences encoding one or more component whose association results in the formation of a FRMS or animals that have been genetically engineered to no longer express endogenous gene sequences encoding one or more component whose association results in the formation of a FRMS (*i.e.*, "knock-out" animals), and their progeny.

Any technique known in the art may be used to introduce an gene encoding one or more component whose association results in the formation of a FRMS transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe and Wagner, 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten, *et al.*, 1985, Proc. Natl. Acad. Sci., USA 82, 6148-6152); gene targeting in embryonic stem cells (Thompson, *et al.*, 1989, Cell 56, 313-321); electroporation of embryos (Lo, 1983, Mol. Cell. Biol. 3, 1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57, 717-723). (For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115, 171-229.)

Any technique known in the art may be used to produce transgenic animal clones containing a one or more component whose association results in the formation of a FRMS transgene, for example, nuclear transfer into enucleated oocytes of nuclei from

cultured embryonic, fetal or adult cells induced to quiescence (Campbell, *et al.*, 1996, Nature 380, 64-66; Wilmut, *et al.*, Nature 385, 810-813).

The present invention provides for transgenic animals that carry a transgene of one or more component whose association results in the formation of a FRMS in all their cells, as well as animals that carry the transgene in some, but not all their cells, *i.e.*, mosaic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (Lasko, *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89, 6232-6236). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the gene transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene encoding one or more component whose association results in the formation of a FRMS in only that cell type, by following, for example, the teaching of Gu, *et al.* (Gu, *et al.*, 1994, Science 265, 103-106). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant component whose association results in the formation of a FRMS gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques that include but are not limited to Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR (reverse transcriptase PCR). Samples of *the* component gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the component transgene product.

In a specific embodiment, the invention encompasses the use of a transgenic mouse for the development of model system which allows screening of a test vaccines intended as vaccines for a species other than mouse. In a preferred embodiment, the test vaccine is a vaccine which is intended for a human.

The transgenic mouse model of the invention encompasses a mouse which is constructed to express the host cell receptors and/or co-receptors of another species for a virus which is not native to a mouse. Such mice are advantageous for the analysis of virus neutralization, since the effect of neutralizing antibodies directed to the host cell receptors is eliminated. These mice provide a model system for viral tolerance. For example, a transgenic mouse may be constructed or used which expresses human host cell receptor(s) for a virus capable of infecting a human (such as HIV).

In one embodiment of the invention, vaccine studies utilize transgenic mice expressing human CD4 and CCR5 co-receptor under the control of CD4 regulatory elements which restrict co-expression to thymocytes and T helper lymphocytes (Killeen et al., 1993, EMBO J., 12:1547-53). These mice are used in order to allow for the analysis of virus neutralization without the confounding effect of neutralizing antibodies directed to CD4 or CCR5. In this regard, the immune response mimics that in humans; immunogenic epitopes are restricted to envelope and to HIV-dependent conformations of CD4 and co-receptor. Although post-entry restrictions to HIV replication have to-date limited the utility of these mice for infectivity studies, the present invention provides a novel use as a system for the analysis of HIV vaccines. In this model, the transgenes function only to provide tolerance to the human CD4 and CCR5 components of the vaccine.

In order to conduct the assay, transgenic mice are immunized one or more times with the FRMS of the invention. Optionally, control immunogens may be used to vaccinate other mice which serve as a control. The FRMS immunogen may be in any of the forms described herein including in cell lysate, in solution, in a cross-linked structure, isolated, purified, etc. The FRMS immunogen may also be introduced to the mouse in a vaccine formulation.

Next, sera or antibodies produced from mice immunized the FRMS or control immunogens are collected and assayed for the ability to neutralized PI virus. Serum is collected at an appropriate time following immunization to allow for the production of an immune response in the mouse. Such times are well known in the art. In one embodiment, sera is obtained 2 weeks following each immunization.

Preferably, sera or antibodies are tested for the ability to neutralize two, three or four primary isolates of the virus. More preferably, sera or antibodies are tested for the ability to neutralize four, six, eight primary isolates of the virus. Most preferably, sera or antibodies are tested for the ability to neutralize 10-15, 15-25, or more primary isolates of the virus.

Neutralization of a primary isolate may be assayed by methods known in the art including those presented herein. In one embodiment, neutralization is determined by the inhibition of infectivity associated with incubating the test antibody with a sample of the

virus to be used in infection. An antibody which inhibits (e.g. decreases or blocks) viral infection, is indicative of a neutralizing antibody. In other embodiments, sera or antibodies are tested for the ability to inhibit syncytia formation or to inhibit infection by cell-free virus and assess the effects of the compound on cell proliferation and viability. In a preferred embodiment of the invention, the test antibody is a monoclonal antibody. In a more preferred embodiment of the invention, the test antibody is a human monoclonal antibody produced in response to a FRMS immunogen. As will be apparent to one skilled in the art, little or no inhibition of infectivity or neutralization activity will be expected to be observed in sera or antibodies obtained from mice immunized with control immunogens.

In other embodiments of the invention, a virus neutralization assay is performed on more than one primary isolates. In preferred embodiments, virus neutralization assay are performed on 2-5, 5-10, 10-15 primary isolates. In a more preferred embodiment, neutralization assay are performed on 15-20, 20-30, or 30 or more primary isolates. In another preferred embodiment, the primary isolates assayed are from more than one viral clade.

Neutralization may characterized in terms of percent neutralization of a number of primary isolates. Generally, in this embodiment, a single assay is used to assess antibody neutralization. In one embodiment, an antibody of the invention neutralizes 30-40%, 40-50%, or 50-60% of the primary isolates of the virus. In a preferred embodiment, an antibody of the invention neutralizes 60-75%, 70-85%, or 80-90% of the primary isolates of the virus. In a most preferred embodiment, an antibody of the invention neutralizes 90-95%, 95-98%, or 99-100% of the primary isolates of the virus. In preferred embodiments, when mAb are tested for neutralization of primary isolates, neutralization is expressed as the number of foci in the presence of mAb (or hybridoma supernatant) relative to the number in the presence of medium alone.

In a specific embodiment, Transgenic mice (hu CD4+, hu CCR5+, mouse CD4+) are immunized with an HIV FRMS immunogen (cross-linked COS-env co-cultured with U87-CD4-CCR5 cells) or with cell controls (U87-CD4-CCR5 cells alone or cocultured with mock-transfected COS cells). Sensitivity of the homologous 168P virus to neutralization by vaccine sera is determined with U87-CD4 cells expressing either CCR5 or CXCR4 co-receptor (*see*, LaCasse, et al., 1998, Science 72:2491; Follis, K.E., et al.,1998, Journal of Virology 72:7603).

In another specific embodiment of the invention, wherein HIV primary isolates are used in a neutralization assay such primary isolates may include but are not limited to 92US657, 92US660, 92TH014, 89.6, 320NSI, 320SI, SHIV89.6P, 168P, 92RW023, 92UG031, 92UG037, 92RW008, 93IN101, 93IN999, 93IN905, 93IN904, 92UG035, 92UG021, 92UG024, 92UG046, 92TH023, 92TH024, 93TH051, and 93TH053.

In one embodiment of the invention, an antibody produced to an HIV FRMS by the methods of the invention (see Section 5.6) is capable of neutralization of 60-70%, 70-80%, 80-85% of such primary isolates of HIV. In a preferred embodiment, an antibody produced to an HIV FRMS is capable of neutralization of 80-90%, 90-95%, or 95-99% of such primary isolates of HIV. In a most preferred embodiment, an antibody produced to an HIV FRMS is capable of neutralization of 100% of such primary isolates of HIV.

In another specific embodiment, primary isolates of HIV may include one or more isolates of clade A, B, C, D, E, F, G, H, or I. In other embodiments, primary isolates of HIV may include one or more isolates an M-group, O-group, or N-group.

In another embodiment, neutralization activity can be demonstrated as antibody-mediated by methods known in the art. For example, sera can be adsorbed to a solid support containing Protein-A and Protein-G. In the case that an antibody is responsible for the virus neutralization activity of the sera, sera depleted of Protein-A and Protein-G binding-proteins will be expected to contain little or no virus neutralization activity, while eluate from the Protein-A and Protein-G solid support will be expected to contain virus neutralization activity.

Accordingly, the present invention provides a method of screening a molecular structure for vaccine efficacy comprising immunizing a transgenic non-human mammal with the molecular structure, wherein said transgenic non-human mammal expresses from one or more transgenes both human CD4 and a co-receptor for HIV, and detecting any neutralizing antibodies to HIV that are produced by said mammal.

The invention also provides a method of screening a molecular structure for vaccine efficacy comprising immunizing a transgenic non-human mammal with the molecular structure, wherein said transgenic non-human mammal expresses from one or more transgenes said one or more host cellular membrane proteins; and detecting any neutralizing antibodies to said virus that are produced by said mammal.

5.7.2. DETERMINATION OF VACCINE EFFICACY

Immunopotency of the one or more immunogens of the invention can be determined by monitoring the immune response of test animals following immunization with the FRMS by use of any immunoassay known in the art. Generation of a humoral (antibody) response and/or cell-mediated immunity, may be taken as an indication of an immune response. Test animals may include mice, hamsters, dogs, cats, monkeys, rabbits, chimpanzees, etc., and eventually human subjects.

As described in Section 5.10 herein, methods of introduction of the vaccine may include oral, intracerebral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal or any other standard routes of immunization. The immune

response of the test subjects can be analyzed by various approaches such as: the reactivity of the resultant immune serum to the immunogen of the invention, as assayed by known techniques, *e.g.*, enzyme linked immunosorbent assay (ELISA), immunoblots, radioimmune assays (RIA) radioimmunoprecipitations, etc. Alternatively, protection of immunized hosts from infection by the pathogen and/or attenuation of symptoms due to infection by the pathogen in immunized hosts can serve as evidence of vaccine efficacy.

As one example of suitable animal testing of viral vaccine, the vaccine of the invention may be tested in rabbits for the ability to induce an antibody response to the FRMS immunogen. Male specific-pathogen-free (SPF) young adult New Zealand White rabbits may be used. The test group each receives a fixed concentration of the vaccine. A control group receives an injection of 1 mM Tris-HCl pH 9.0 without the FRMS immunogen. Blood samples may be drawn from the rabbits every one or two weeks, and serum analyzed for antibodies to FRMS. The presence of antibodies specific for the immunogen may be assayed, e.g., using an ELISA.

In a preferred embodiment of the invention, animal testing of viral vaccine effacy is by use of a transgenic mouse model as described herein in Section 5.7.1.

The invention also provides methods for optimizing and quantitating vaccines comprising the subject FRMS. Preferably, the subject complexes are prepared using autologous cells negating reactivity toward host cellular components. Vaccine compositions comprising complexes prepared using non-autologous cells, however, can be assessed using transgenic animals genetically engineered to be tolerant to the host cellular components of the complexes. Using these transgenic animals it is possible to determine whether the success achieved by vaccinating with the fusion-complexes of the subject invention is due to novel fusion-competent epitopes presented by the complexes. For example, in one preferred embodiment, the FRMS result from the interaction of HIV(168P) viral envelope protein and the human proteins CD4 and CCR5. As described in Section 6, vaccine compositions have been tested in transgenic mice genetically engineered to be tolerant to the human components of the vaccine. Mice expressing both the human CD4 receptor and the human CCR5 co-receptor were used to evaluate the complex and demonstrate that the neutralizing antibody generated was to immunologic epitopes restricted to envelope protein or HIV-dependent conformations of CD4 and co-receptor. It is noted that these transgenic mice were originally used as a mouse model for HIV infection. Utilizing transgenic animals to screen vaccine immunogens is a novel use of these mice. Transgenic mice exhibiting tolerance to host portions of vaccines can be used to optimize vaccine components, test variants and evaluate adjuvants.

Further, it should be apparent to one skilled in the art that this principal can be applied to any viral model to provide rapid and safe methods for screening viral

vaccines. Additionally, it should be apparent that transgenic animals other than transgenic mice can be used to screen candidate vaccines.

In one embodiment, the invention provides a method of screening a molecular structure for vaccine efficacy comprising immunizing a transgenic non-human mammal with a molecular structure comprising an epitope formed as a result of association of (a) an envelope protein of an enveloped virus, with (b) one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins, wherein said transgenic non-human mammal expresses from one or more transgenes said one or more host cellular membrane proteins; and detecting any neutralizing antibodies to said virus that are produced by said mammal. In a preferred embodiment, the molecular structure is isolated.

In a specific embodiment, the invention provides a method of screening a molecular structure for vaccine efficacy comprising immunizing a transgenic non-human mammal with a molecular structure comprising an epitope formed as a result of association of (a) an HIV envelope protein, or a mutant thereof that assembles into the viral envelope; with (b) human CD4 and a co-receptor for HIV fusion, wherein said transgenic non-human mammal expresses from one or more transgenes both human CD4 and a co-receptor for HIV, and detecting any neutralizing antibodies to HIV that are produced by said mammal. In a preferred embodiment, the mammal is a mouse. In another preferred embodiment, the molecular structure is isolated.

5.8. USES FOR ANTIBODIES

The antibodies generated by the vaccine formulations of the present invention antibodies can be used in methods known in the art. For example, the antibodies generated against the FRMS or vaccine immunogen by immunization with the FRMS of the present invention also have potential uses such as diagnostic immunoassays, passive immunotherapy, viral decontamination, anti-viral treatment and prevention and generation of antiidiotypic antibodies.

The subject invention also encompasses antibodies that are elicited by the novel epitopes of the FRMS of the subject invention contain antibodies useful as reference standards and components in diagnostic assays and kits. Additionally, mAb to the FRMS of the subject invention can be used as reference standards, diagnostic agents for *in vitro* and *in vivo* bioassays and as markers for critical determinants in binding and fusion. For example, during large scale production of the FRMS of the invention, the mAb of the invention are useful in determining the amount of FRMS produced in each batch or lot. In this embodiment, the mAb may be used to determine the amount to lot-to-lot variation (*e.g.*,

by ELISA, or RIA using the mAb). Alternatively, labeled FRMS may be used, by way of a competition assay with the test FRMS for binding a mAb specific for the FRMS.

Similar assays may be used to determine the antibody titer of a sample of serum from a subject. In such an assay, a sample of serum from a subject is taken at a time following administration of the vaccine of the invention. The sample serum is then tested for ability to neutralize virus. The serum may also be tested for the ability to bind to the immunogen FRMS, or to compete with a neutralizing mAb for binding to the immunogen FRMS. The inability or weak ability of the serum sample to neutralize virus or bind FRMS provides an indication that booster vaccinations with the FRMS immunogen are desired for the subject. Accordingly, the present invention provides a method for monitoring the production of antibody to the molecular structure in a subject previously administered an amount of the molecular structure comprising isolating from said subject a sample comprising serum; and detecting the presence of any antibodies to the molecular structure in said serum. In one embodiment, detecting is carried out by a method comprising performing a competitive immunoassay with labeled antibody to the molecular structure.

Further, polyclonal sera or monoclonal antibodies can be administered to individuals for purposes of treatment or prevention of viral infection and its sequences. In particular embodiments, such antibody can be administered for purposes post-exposure prophylaxis or to prevent maternal transfer of virus to the neonate by way of passive immunization. Humanized mAb of the invention can be used in protection against maternal-infant virus transmission or in post-exposure prophylaxis or treatment. MAb of the present invention are also useful in passive immunization studies in a hu-PBL-scid mouse model of HIV infection and in SHIV studies in rhesus macaques. Accordingly, the present invention provides a method for treating or preventing infection by HIV in a human fetus comprising administering to a pregnant human containing said fetus an amount of the monoclonal antibody effective to treat or prevent infection by HIV in said fetus.

Production of mAb to the FRMS of the subject invention can be used to further delineate the biochemical and immunochemical pathway to infection, and define critical epitopes for PI virus neutralization. Monoclonal antibodies (mAb) generated to the FRMS of the subject invention can be used to dissect the biochemical basis for binding and entry, and the immunochemical basis for primary isolate virus neutralization. In this embodiment, immunogens should be chosen for MAb development that excel in immunogenicity studies in vaccination models, are potent and elicit a broad range of primary isolate virus neutralizing antibodies.

The antibodies of the present invention can also be used in the production of antiidiotypic antibody. The antiidiotypic antibody can then in turn be used for immunization, in order to produce a subpopulation of antibodies that bind the initial antigen

of the pathogenic microorganism (Jerne, 1974, Ann. Immunol. (Paris) 125c:373; Jerne, et al., 1982, EMBO J. 1:234).

Further, mAb can also be used as an anti-viral agent in blood and blood products. The present invention provides neutralizing antibodies that are useful as an additive for the decontamination of blood or blood products which are contaminated or suspected of being contaminated with a virus. For example, the mAb of the invention is useful as an additive to donor blood (*e.g.*, blood within a blood bank) which is to be used in the treatment of a subject. The mAb of the invention is useful as an additive to blood collection bags, gloves, blood sample collection containers, etc. In a specific embodiment of the embodiment, one or more mAb(s) of the invention are used to cleanse the birth canal prior to delivery of a child in order to prevent perinatal infection. Effective amounts of the mAb of the invention for blood and blood-product additives include 1μg/ml to 10mg/ml. As will be apparent to one skilled in the art, effective amounts will be influences by antibody affinity, additives, and formulations. Any amount may be used such that the effective amount is capable of inhibiting (e.g. reducing or preventing) viral infection.. In a preferred embodiment, a mixture of neutralizing mAb against a variety of viruses is used in decontamination. In a specific embodiment, an effective amount of a neutralizing mAb of the invention which is immunospecific for HIV is added to a sample of human blood before such blood is transfused into a recipient subject. In this embodiment, the HIV is neutralized by the mAb. Accordingly, the present invention provides a sample of mammalian blood, to which an amount of the antibody has been added effective to inhibit or decrease infection by the virus. In a specific embodiment, the present invention provides a sample of human blood, to which an amount of the antibody has been added effective to inhibit or decrease infection by HIV. The present invention also provides a method of inhibiting infection by a virus in a sample of blood comprising contacting said sample of blood with an amount of the monoclonal antibody effective to inhibit infection by said virus. In a specific embodiment, the present invention provides a method of inhibiting infection by HIV in a sample of human blood comprising contacting said sample of human blood with an amount of the monoclonal antibody effective to inhibit infection by HIV.

The neutralizing antibodies of the invention are also useful in decontaminating any object exposed to the virus to which the antibody is directed. Objects which may be decontaminated with the mAb of the invention include but are not limited to surgical and dental tools (such as drills, picks, scalpels, etc). In a preferred embodiment, a mixture of neutralizing mAb against a variety of viruses is used in decontamination. In a preferred embodiment, objects which are not readily disposable (*e.g.*, computer aided tools, robotic aided tools, specialized tools, etc.) are decontaminated with one or more mAbs of the invention. Effective amounts of the mAb of the invention for decontamination include

1μg/ml to 10mg/ml. As will be apparent to one skilled in the art, effective amounts will be influences by antibody affinity, additives and formulations. Any amount may be used such that the effective amount is capable inhibiting viral infection following decontamination. In a specific embodiment, HIV is neutralized in the decontamination. Accordingly, the present invention provides a method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody effective to inhibit infection by said virus. In a specific embodiment, the present invention provides a method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody effective to inhibit infection by HIV.

In another embodiment of the invention antibodies directed to an FRMS may be used to screen molecules which comprise an epitope recognized by the antibody. For example, a mAb of t he invention can be used to screen small molecules for the presence of an antibody-recognized epitope. In one binding of the mAb to the small molecules indicates the presence of such epitope on the small molecule. Such small molecules in turn may be tested as potential immunogens or therapeutics. In another embodiment, the mAbs of the invention may be used to identify peptides which bind to the antibody (e.g., from a phage display library). Such peptides may be tested, for example, for immunogenicity (i.e. as mimetopes).

The mAb of the invention are also useful as an anti-viral agent in contraceptive or microbicide products. The addition of one or more mAb of the invention to a contraceptive or microbicide product is particularly preferred for viruses which are known to be sexually transmitted. Thus, the invention provides a contraceptive product comprising an effective amount of one or more neutralizing mAb(s) of the invention. Contraceptive or microbicide products to which the mAb of the invention may be added include but are not limited to creams, foams, jellies, ointment, condoms, diaphragms, etc. The contraceptive products may further comprise of spermicidal agent. In a preferred embodiment, a mixture of neutralizing mAbs against a variety of viruses is used in the contraceptive product. Effective amounts of the mAb of the invention for contraceptive or microbicide products include 1μg/ml to 10mg/ml. As will be apparent to one skilled in the art, effective amounts will be influences by antibody affinity, additives and formulations. Any amount may be used such that the effective amount is capable of inhibiting (e.g. reducing or preventing) viral infection. For example, for the use in an anti-viral contraceptive, an effective amount is that which is capable of inhibiting infection upon exposure by sexual transmission of a virus. In a specific embodiment, the contraceptive or microbicide products comprises mAb of the invention immunospecific to HIV or other sexually transmitted virus. Accordingly, the present invention provides a contraceptive or microbicide in the form of a jelly, foam, cream, or ointment comprising an amount of the

antibody of the invention effective to inhibit or decrease infection by the virus. In a specific embodiment, the present invention provides a contraceptive or microbicide in the form of a jelly, foam, cream, or ointment comprising an amount of the antibody of the ineffective to inhibit or decrease infection by HIV.

5.9. THERAPEUTIC COMPOSITIONS

The present invention provides methods of eliciting production of anti-FRMS antibodies in a subject by the administration of a FRMS. Any of the FRMS of the invention, and functionally active fragments, analogs, and derivatives thereof; nucleic acids encoding the FRMS of the invention, and functionally active fragments and derivatives thereof; as well as antibodies which immunospecifically bind to a FRMS may be used as therapeutic, (termed herein "Therapeutic").

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the Therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together

with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

5.10. GENE THERAPY

Gene therapy refers to therapy performed by the administration of a nucleic acid molecule to a subject. In this embodiment of the invention, the nucleic acid molecule(s) produces its encoded protein(s) and mediates a therapeutic effect by forming a FRMS in vivo.

In a specific embodiment, a nucleic acid molecule comprising a sequence encoding one or more components whose association results in the FRMS of the invention or a functional derivative thereof, are administered to produce an immunological response to the FRMS, by way of gene therapy. In more specific embodiments, a nucleic acid or nucleic acids encoding a viral envelope protein of an envelope virus, a host cell receptor, and a host cell co-receptor or functional derivatives thereof, are administered by way of gene therapy. In other embodiment, a viral envelope protein of an envelope virus administered by way of gene therapy.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; and May, 1993, TIBTECH 11:155-215). Methods commonly known in the art for recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY) and Kriegler, 1990, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY.

In a preferred specific embodiment, the Therapeutic comprises an HIV envelope protein and a human CD4 receptor, and a co-receptor (such as a chemokine receptor CCR5 or CXCR4) in a suitable host. In another particular embodiment, a nucleic acid molecule is used in which the HIV envelope protein coding sequence is delivered to a subject, which subject expresses native CD4 and a native co-receptor.

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* and *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see, U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), which can be used to target cell types specifically expressing the receptors, etc. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide that disrupts endosomes, preventing lysosomal degradation of the nucleic acid. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression by targeting a specific receptor (see, *e.g.*, International Patent Publications WO 92/06180 by Wu et al., WO 92/22635 by Wilson et al., WO 92/20316 by Findeis et al., WO 93/14188 by Clarke et al., and WO 93/20221 by Young). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by

homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935, Zijlstra et al., 1989, Nature 342:435-438).

In a specific embodiment, a viral vector that contains a nucleic acid encoding a viral envelope protein, and host cellular membrane protein(s) (e.g., host cell receptor(s)) are used. Any of the viral vectors described in Section 5.1.2, may be used for gene therapy purposes. For example, a retroviral vector can be used (see Miller et al., 1993, Meth. Enzymol. 217:581-599). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. Other references illustrating the use of retroviral vectors in gene therapy include: Clowes et al., 1994, J. Clin. Invest. 93:644-651, Kiem et al., 1994, Blood 83:1467-1473, Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141, and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

As described in Section 5.1.2, Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, Current Opinion in Genetics and Development 3:499-503 present a review of adenovirus-based gene therapy. Bout et al., 1994, Human Gene Therapy 5:3-10, demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, Science 252:431-434, Rosenfeld et al., 1992, Cell 68:143-155, and Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300).

Another approach to gene therapy involves transferring a gene into cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Any method known in the art to transfer a gene into a cell may be used in connection with the gene therapy aspect of the invention, including but not limited to those described in Section 5.1.2. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection,

electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618, Cohen et al., 1993, Meth. Enzymol. 217:618-644, Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell, and is heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. In a preferred embodiment, epithelial cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the patient. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes, and blood cells, such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, a nucleic acid molecule encoding the components whose association results in the formation of a FRMS is used. In a specific embodiment, for example, the nucleic acid encodes an HIV viral envelope protein, human CD4 and a co-receptor (such as a chemokine receptor CCR5 or CXCR4). The encoding nucleic acid molecule, is/are introduced into the cells such that the gene or genes are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention. Such stem cells include but are not limited to hematopoietic stem cells (HSC), stem cells of epithelial tissues such as the skin and the

lining of the gut, embryonic heart muscle cells, liver stem cells (International Patent Publication WO 94/08598), and neural stem cells (Stemple and Anderson, 1992, Cell 71:973-985).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region (see Section 5.1.2), such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

Additional methods can be adapted for use to deliver a nucleic acid molecule encoding the components which result in the formation of the FRMS of the invention will be apparent to one skilled in the art, and are within the scope of the invention.

Accordingly, the invention provides a method of treating or preventing infection by a virus in a subject comprising administering to the subject (a) a first nucleic acid encoding an envelope protein of an enveloped virus; and (b) a second nucleic acid encoding one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins, such that the envelope protein and cellular membrane proteins are expressed in the subject and neutralizing antibodies to the virus are produced. In one embodiment, the method wherein the first and second nucleic acids are the same. In another embodiment, the first and second nucleic acid are different nucleic acid vectors. In a specific embodiment, the envelope protein is en envelope protein of HIV, and the cellular membrane proteins are CD4 and an HIV co-receptor.

5.11. METHODS OF ADMINISTRATION

The invention provides methods of treatment and prevention of viral infection and disease by administration to a subject in need of such treatment of a therapeutically or prophylactically effective amount of a Therapeutic of the invention. The subject is preferably an animal, including, but not limited to, animals such as monkeys, cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, the subject is a human not afflicted with HIV infection.

Various delivery systems are known and can be used to administer a Therapeutic of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the Therapeutic, receptor-mediated endocytosis (*see, e.g.*, Wu et al., 1987, J. Biol. Chem. 262:4429-4432), construction of a Therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous,

subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention comprising antibody into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention comprising antibody locally to the area in need of treatment; this may be achieved, for example and not by way of limitation, by topical application, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In a highly specific embodiment, the pharmaceutical compositions of the invention is administered to an open wound of a human, which wound is suspected of being exposed to HIV.

In another embodiment, the Therapeutic can be delivered in a vesicle, in particular a liposome (*see*, Langer, 1990, Science 249:1527-1533; Treat et al., 1989 in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365; Lopez-Berestein, *Science.*, pp. 317-327; *see* generally *Science.*)

In yet another embodiment, the Therapeutic can be delivered in a controlled release system. In one embodiment, a pump may be used (*see* Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980, Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger et al., 1983, J. Macromol. Sci. Rev. Macromol. Chem. 23:61; *see also* Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351; Howard et al., 1989, J. Neurosurg. 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, in Medical Applications of Controlled Release, 1984, *supra*, vol. 2, pp. 115-138).

Other controlled release systems are discussed in the review by Langer (Langer, 1990, Science 249:1527-1533).

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition.

5.12. DEMONSTRATION OF UTILITY AGAINST HIV

The present invention provides assays to test the anti-HIV efficacy of antibodies directed to an HIV-FRMS. The invention further provides methods to monitor FRMS vaccine efficacy in patients or subjects by assaying antibodies in a sample of sera derived from the patient or subject following vaccination The Therapeutics of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. Any *in vitro* or *in vivo* assay known in the art to measure viral infection or production can be used to test the efficacy of a Therapeutic of the invention.

In an embodiment of the invention, a method of screening a preparation comprising a mAb for anti-HIV activity is provided, which assay comprises assaying said preparation or fraction for the ability to inhibit HIV infection.

By way of example, to assay a Therapeutic *in vitro*, one can examine the effect of the Therapeutic on HIV infection in cultured cells. Briefly, cultured hematopoietic cells (*e.g.*, primary PBMCs, isolated macrophages, isolated $CD4^+$ T cells or cultured H9 human T cells) are acutely infected with HIV-1 using titers known in the art to acutely infect cells *in vitro*, such as 10^5 $TCID_{50}$/ml. Then, appropriate amounts of the Therapeutic are added to the cell culture media. Cultures are assayed 3 and 10 days after infection for HIV-1 production by measuring levels of HIV antigen using an ELISA assay. Reduction in HIV antigen levels over levels observed in untreated controls indicates the Therapeutic is effective for treatment of HIV infection. Alternatively, any commercially available HIV ELISA may be used as a comparative assay for pre- and post- treatment with the Therapeutic.

Additionally, assays for HIV-1 LTR driven transcription are useful for testing the efficacy of Therapeutics of the invention. Specifically, a reporter gene, *i.e.*, a gene the protein or RNA product of which is readily detected, such as, but not limited to, the gene for chloramphenicol acetyltransferase (CAT), is cloned into a DNA plasmid construct such that the transcription of the reporter gene is driven by the HIV-1 LTR promoter. The resulting construct is then introduced by transfection, or any other method

known in the art, into a cultured cell line, such as, but not limited to, the human $CD4^+$ T cell line HUT 78. After exposure of the transformed cells to the Therapeutic, transcription from the HIV-1 LTR is determined by measurement of CAT activity using techniques which are routine in the art. Reduction in HIV-1 LTR driven transcription demonstrates utility of the Therapeutic for treatment and/or prevention of HIV infection.

Exemplary tests in animal models are described in Section 5.7.1. herein.

The efficacy of Therapeutics of the invention can also be determined in SIV infected rhesus monkeys (*see*, Letrin, N.L., et al., 1990, J. AIDS 3:1023-1040), particularly rhesus monkeys infected with SIV_{mac251}, which SIV strain induces a syndrome in experimentally infected monkeys which is very similar to human AIDS (Kestler, H., et al., 1990, Science 248:1109-1112). Specifically, monkeys can be infected with cell free SIV_{mac251}, for example, with virus at a titer of $10^{4.5}$ $TCID_{50}$/ml. Infection is monitored by the appearance of SIV p27 antigen in PBMCs. Utility of the Therapeutic is characterized by normal weight gain, decrease in SIV titer in PBMCs and an increase in $CD4^+$ T cells. Alternatively, a SHIV model is used in which an HIV env protein is constructed into the backbone of SIV. In one embodiment, monkeys are immunized using a FRMS vaccine comprising cells expressing HIV env and cells expressing rhesus CD4 and CCR5 coreceptor. If adequate *in vitro* neutralization is obtained, then animals will be challenged with an infectious SHIV virus bearing a neutralization-sensitive HIV env. Specifically, monkeys are vaccinated with an immunogen comprising one or more FRMS and challenged with SHIV 89.6P primary isolate (see e.g., Montefiori et al., 1998, J Virol. 72:3427-31).

Once the Therapeutic has been tested *in vitro*, and also preferably in a non-human animal model, the utility of the Therapeutic can be determined in human subjects. The efficacy of treatment with a Therapeutic can be assessed by measurement of various parameters of HIV infection and HIV associated disease. Specifically, the change in viral load can be determined by quantitative assays for plasma HIV-1 RNA using quantitative RT-PCR (Van Gemen, B., et al., 1994, J. Virol. Methods 49:157-168; Chen, Y.H., et al., 1992, AIDS 6:533-539) or by assays for viral production from isolated PBMCs. Viral production from PBMCs is determined by co-culturing PBMCs from the subject with non-infected PBL cells and subsequent measurement of HIV-1 titers using an ELISA assay for p24 antigen levels (see e.g.,Wrin, T., et al., 1995, *Science* 69:39; Popovic, M., et al., 1984, Science 204:497-500). Administration of the Therapeutic can also be evaluated by assessing changes in $CD4^+$ T cell levels, body weight, or any other physical condition associated with HIV infection or AIDS or AIDS Related Complex (ARC). Reduction in HIV viral load or production, increase in $CD4^+$ T cell or amelioration of HIV-associated symptoms demonstrates utility of a Therapeutic for administration in treatment/prevention of HIV infection.

5.13. KITS

The invention also concerns kits comprising the fusion-competent protein complexes or antibodies generated by those complexes. Kits including the subject complexes or antibodies can be used to analyze vaccine lots, conduct stability studies or develop vaccine release qualifications. Antibodies and the complexes can also be used in diagnostic kits to detect the presence of antibody or virus in the host system. For example, antibodies or the subject complexes can be used as substrate or reagent in a kit providing a standard diagnostic enzyme-linked immunosorbent assay (ELISA) or competitive ELISA.

The invention also provides a pharmaceutical pack or kit comprising one or more containers comprising one or more of the ingredients of the vaccine formulations of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

In one embodiment, the invention provides a kit comprising in one or more containers a labeled monoclonal antibody to a molecular structure comprising an epitope formed as a result of association of (a) an envelope protein of an enveloped virus, with (b) one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins. In a further embodiment, the invention provides a kit which further comprises in a separate container a molecular structure comprising an epitope formed as a result of association of (a) an envelope protein of an enveloped virus, with (b) one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins.

In a specific embodiment, the invention provides a kit comprising in one or more containers a labeled monoclonal antibody to a molecular structure comprising an epitope formed as a result of association of (a) an HIV envelope protein, or a mutant thereof that assembles into the viral envelope; with (b) human CD4 and a co-receptor for HIV fusion. In a further embodiment, the invention provides a kit which further comprises in a separate container an epitope formed as a result of association of (a) an HIV envelope protein, or a mutant thereof that assembles into the viral envelope; with (b) human CD4 and a co-receptor for HIV fusion.

In one embodiment, the invention provides a kit comprising in one or more containers, a molecular structure comprising an epitope formed as a result of association of (a) an envelope protein of an enveloped virus, with (b) one or more cellular membrane

proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins. In a preferred embodiment, the molecular structure is isolated. In a further embodiment, the invention provides a kit which further comprises a pharmaceutically acceptable carrier, and wherein said molecular structure is present in an immunological amount.

In another embodiment, the invention provides a kit comprising in one or more containers (a) a first nucleic acid encoding an envelope protein of an enveloped virus; and (b) a second nucleic acid encoding one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins, such that the envelope protein and cellular membrane proteins are expressed in the subject and neutralizing antibodies to the virus are produced.

6. EXAMPLES

The inventors of the present invention have made the surprising discovery that the ability to neutralize PI viruses is related to the presentation of functioning envelope protein in active infection, as compared to the static, non-functioning presentation of the envelope protein in rgp120 vaccines.

As described herein, the HIV envelope protein orchestrates a complex series of protein-protein interactions and structural changes that ultimately results in fusion of the virus and cell membranes, and infection of the cell. Upon binding to CD4, the envelope protein undergoes conformational change that facilitates subsequent interaction with one of several co-receptor molecules, predominantly the CC chemokine receptor 5 (CCR5) or the CXC chemokine receptor 4 (CXCR4) (Berger, E.A., 1997, AIDS 11 (suppl A), S3; Moore, J.P., et al., 1997, Current Opinions in Immunology 9:551; Doranz, B.J., et al., 1997, Immunology Research 16:15). Without limitation as to mechanism, interaction with either co-receptor induces further conformational change in the envelope protein and exposure of the hydrophobic fusion domain of the transmembrane gp41 subunit, which then mediates fusion of the apposed cell and virus membranes. On the basis of this dynamic model of HIV binding and entry, HIV vaccine immunogens were developed by the methods of the invention that explicitly incorporate these functional intermediate structures.

The examples described herein serve to illustrate the methods of and compositions of the invention.

Example 1:Broad Primary Isolate Neutralization from HIV-FRMS Immunogen

As one example of the invention, HIV vaccine immunogens were generated that capture the transient structures that arise during HIV binding and fusion. In a transgenic mouse immunization model, formaldehyde-fixed whole cell vaccines elicited antibodies capable of neutralizing infectivity of 23 of 24 primary HIV isolates from diverse geographic locations and genetic clades A-E. Development of these fusion-dependent immunogens provide clinically important and broadly effective HIV vaccines.

As described herein, the HIV envelope protein orchestrates a complex series of protein-protein interactions and structural changes that ultimately results in fusion of the virus and cell membranes, and infection of the cell. Without limitation as to mechanism, upon binding to CD4, the envelope protein undergoes conformational change that facilitates subsequent interaction with one of several co-receptor molecules, predominantly the CC chemokine receptor 5 (CCR5) or the CXC chemokine receptor 4 (CXCR4) (Reviewed in Berger, E.A., 1997, AIDS 11(suppl A), S3; Moore, J.P., et al., 1997, Current Opinions in Immunology 9:551; Doranz, B.J., et al., 1997, Immunology Research 16:15). Interaction with either co-receptor induces further conformational change in the envelope protein and exposure of the hydrophobic fusion domain of the transmembrane gp41 subunit, which then mediates fusion of the apposed cell and virus membranes. The present example illustrate methods to develop HIV vaccine immunogens that explicitly incorporate these functional intermediate structures.

One measure of envelope protein function is the ability to mediate cell-cell fusion. When cells expressing envelope protein are cocultured with cells expressing CD4 and co-receptor, multinucleated syncytia form over the course of 6-24 hr. In the instant example, the process of binding and fusion was captured in progress by formaldehyde-crosslinking prior to extensive syncytium-formation.

Preparation of the FRMS

In these studies, the functioning envelope protein was derived from a T-lymphocytropic PI virus obtained from the Amsterdam Cohort (ACH168.10; 168P). The molecularly cloned envelope gene of ACH168.10 was isolated by PCR using the pCR3.1-Uni plasmid (Invitrogen) (Tersmette, M., et al., 1989, Journal of Virology 63:2118; Wrin, T., et al., 1995, *Science* 69:39; LaCasse, R.A., et al., 1998, Journal of Virology 72:2491). The molecularly cloned envelope protein, as well as the parental syncytium-inducing(SI) virus, utilizes both CCR5 and CXCR4 co-receptors.

Cos-7 cells were transfected to express the envelope protein (COS-env) and subsequently cocultured with human U87 glioma cells that express CD4 and CCR5 co-receptor (U87-CD4-CCR5). The functional 168P (168P23) envelope gene was transfected

into COS-7 cells (American Type Culture Collection, Manassus, VA) by calcium phosphate precipitation (20 μg DNA/10^6 cells/10 cm culture dish) by methods known in the art (*see e.g.*, Jordan, M., et al., 1996, Nucleic Acids Research 24:596). Transiently-expressing COS-7 cells were harvested 2 days later using 0.5 mM EDTA in phosphate-buffered saline (PBS), and U87-CD4-CCR5 fusion partners (Hill, C.M., et al., 1997, Journal of Virology 71:6296) were prepared using 0.1 mM EDTA in PBS. Cocultures were initiated by mixing the two cell types ($1.5x10^6$ cells each) in 10 cm culture dishes. The time course of cell-cell fusion was monitored microscopically and by immunochemical staining (HIVIG) in parallel cocultures (LaCasse, R.A., et al., 1998, *Science* 72:2491). Cocultures were typically harvested by formaldehyde fixation at 4-5 hrs, when little or no overt syncytium formation was evident.

Capturing the FRMS

To capture transitional intermediates during the process of binding and fusion, cocultures were fixed in 0.2% formaldehyde after 5 hrs when few if any multinucleate cells were evident. Specifically, cultures were fixed *in situ* using 0.2% formaldehyde in PBS at 4° overnight by methods known in the art (*see e.g.*, Yamamoto, J.K., et al., 1991, AIDS Research and Human Retroviruses 7:911; Verschoor, E.J., et al., 1995, Veterinary Immunology and Immunopathology 46:139). Cells were subsequently scraped, washed twice with PBS, resuspended at a nominal density of $3x10^6$ cells/0.1 ml in PBS containing 10% DMSO, and frozen at -80° for storage or used immediately as immunogens, as described herein below.

Immunization with a FRMS Immunogen

The formaldehyde treated whole cell preparation was used as a fusion-competent (FC) or FRMS immunogen. To test the ability of these immunogens to elicit neutralizing antibodies, it was necessary to restrict the immune response to viral and virus-induced epitopes. Otherwise, antibodies to CD4 and CCR5 would be generated that would themselves block infectivity. Therefore, an animal model was used that was immunologically tolerant to the human (hu) CD4 and CCR5 components of the vaccine. Thus, immunogenicity studies were performed with transgenic mice that express hu CD4 and hu CCR5 co-receptor.

Construction of a CD4 targeted-deletion and hu CD4 transgenic mouse has been described (Killeen, N., et al., 1993, EMBO Journal 12:1547). The design of a hu CCR5 transgenic mouse is summarized as follows: a 1.15 kb hu CCR5 cDNA was molecularly cloned into an engineered SalI site in exon 2 of a murine CD4 expression cassette (construct c in (Sawada, et al., 1994, Cell 77:917). This minigene contained the

murine CD4 enhancer, the CD4 promoter, the first (noncoding) exon, and intron 1 with an internal deletion that eliminated the CD4 silencer. Transgenic founders were identified by flow cytometry using a mAb by methods known in the art. These animals were bred to hu CD4 expressing transgenic mice to yield progeny expressing hu CD4, hu CCR5, and mouse CD4. Pups were screened for expression of hu CD4, hu CCR5, and mouse CD4 by flow cytometry using a Coulter EPICS ELITE flow cytometer. The following antibody reagents were used: mouse α-human CD4/CyChrome (Pharmingen), mouse α-human CCR5 MAB 180 (R&D Systems) with goat α-mouse Ig/FITC (Caltag), and rat α-mouse CD4 L3T4/PE.

Transgenic mice expressing hu CD4, hu CCR5, and mouse CD4 were immunized with either FRMS immunogen or with cell controls (U87-CD4-CCR5 cells, alone or cocultured with mock-transfected COS cells). Vaccines comprised formaldehyde-fixed whole cells ($3x10^6$ cells/0.1 ml) formulated with an equal volume of Ribi adjuvant (R-700; reconstituted in half the recommended volume of PBS); in some experiments, the initial immunization was with adjuvant containing cell wall material (R-730). Mice received 0.05 ml vaccine in four subcutaneous sites. Booster immunizations were at three week intervals, and mice were bled at 10-28 days post-immunizations from the tail. Serum antibodies directed to gp120 were quantitated by gp120 ELISA as per methods known in the art (*see*, Moore, J., et al., 1989, AIDS 3:155).

Virus Neutralization

Sensitivity of the homologous 168P virus to neutralization by vaccine sera was determined with U87-CD4 cells expressing either CCR5 or CXCR4 co-receptor. This PI virus neutralization assay has been validated relative to standard neutralization assay in PBL culture (LaCasse, R.A., et al., 1998, *Science* 72:2491; Follis, K.E., et al., 1998, Journal of Virology 72:7603) and was determined to perform well in the presence of mouse serum. All sera were heat-inactivated prior to use in neutralization assays.

Neutralization assay of the homologous 168P PI virus by fusion-competent (FC) and fusion-incompetent (FI) vaccine sera was performed as follows. Transgenic mice (hu CD4+, hu CCR5+, mouse CD4+) were immunized with FC immunogen (COS-env with U87-CD4-CCR5; squares; n = 3 mice) or with cell controls (U87-CD4-CCR5 cells alone or cocultured with mock-transfected COS cells; circles n = 3 mice). Unimmunized mice were also used (triangles; n = 2 mice). Sera were tested for neutralization of 168P using U87-CD4 cells expressing either CXCR4 (black symbols) or CCR5 (white symbols). Data represent averages of three to six neutralization assays using serum obtained 2 weeks following second and third immunization.

As indicated in Fig 1, results of virus neutralization assays indicated that no inhibition of infectivity was observed in sera from mice immunized with cell controls,

indicating that the transgenic mice were in fact tolerant to hu CD4 and CCR5 and that other adventitious cellular reactivities did not interfere with the virus infectivity assay (Fig 1). Sera from mice immunized with FRMS immunogens neutralized the homologous 168P PI virus. Neutralization activity was further demonstrated to be antibody-mediated and as such activity could be adsorbed to, and subsequently eluted from, a solid support containing Protein-A and Protein-G . Specifically, serum was adsorbed sequentially to Protein-A Sepharose (Sigma) and Protein-G agarose (Sigma) at 4°. Adsorption of antibody was confirmed by gp120 ELISA. The solid supports were combined and antibodies were eluted using 100 mM glycine pH 2.5. The eluate was neutralized and dialyzed by centrifugal ultrafiltration (Microcon-100; Amicon). Neutralization by FC sera in PBL assay was determined to be >99%, while neutralization by FC sera in U87-CD4-coR cell assay was determined to be 80-90%.

It is interesting to note that in the sensitive PBL neutralization assays 'fusion-incompetent' vaccine sera did demonstrate some inhibition of PI virus replication, albeit far less than 'fusion-competent' vaccine sera (see, LaCasse, RA, et al.,1999, Science 283:357-62.) This minimal effect may account for intermittent claims in the literature of PI virus-neutralization by rgp120 vaccine sera (Devico, A, A Silver, et al., 1996, Virology 218: 258-63;Berman, PW, et al.,1998, AIDS Res & Hum Retrovirus 14 (suppl 3) S277-S89). The molecular basis for differences in neutralization sensitivity among PI viruses is unknown at this time, and may or may not correlate with phylogenetic clade groupings per se. Serotypically-distinct determinants may arise independently in multiple clades. Thus, 'fusion-competent' envs derived from clades other than B may be used to determine empirically the number of prototypic envs that define 'fusion-competent' neutralization serotypes. Regardless of the total number of HIV serotypes, it is significant that 80% of PI viruses tested could be strongly neutralized (>70%) using a single representative but appropriately presented clade B env.

CXCR4 co-receptor may substitute for CCR5

Neutralization of the 168P virus by FC serum was observed regardless of the co-receptor used in the U87-CD4 cell infection assay (Fig 1). Several reports have demonstrated that in general neutralization sensitivity is independent of specific co-receptor use (LaCasse, R.A., et al., 1998, Science 72:2491); Trkola, A. et al., 1998, Journal of Virology 72:1876; Montefiori, D.C., et al., 1998, Journal of Virology 72:3427; Follis, K.E., et al., 1998, Science 72:7603).

The fact that neutralization was observed herein with CXCR4, a co-receptor to which the animal had not been exposed, argues that neutralization may not directly target

the CCR5 component of the vaccine. Further, the CXCR4 co-receptor may substitute for CCR5.

Fusion-Incompetent Immunogens

The role of fusion-dependent determinants in the induction of PI virus neutralization was examined. Fusion-incompetent (FI) immunogens - cocultures that express env but do not undergo cell-cell fusion. These include COS-env cocultured with U87 cells (no CD4 or CCR5 co-receptor), COS-env cocultured with U87-CD4 cells, and COS-env cells to which soluble CD4 (sCD4) was complexed. Specifically, envelope-expressing cultures were incubated with sCD4 (Berger, E.A., et al., 1988, Proceedings of the National Academy of Sciences USA 85:2357) (5 μg/ml; 1 hr at 37°) and subsequently washed to remove unbound sCD4. All FI immunogens were fixed with formaldehyde as above. An additional FI immunogen comprised COS-env and U87-CD4-CCR5 cells that were separately fixed with formaldehyde prior to mixing during the formulation of the vaccine.

In marked contrast to FC or FRMS immunogens, all FI immunogens were unable to elicit significant neutralization of the homologous PI virus (Fig 2A). Specifically, P168 was neutralized by FC, but not FI immunogens. As shown in Fig 2A, transgenic mice were immunized with FC immunogen (black squares; n = 4), FI immunogens (COS-env with U87 cells; gray circles n = 4; COS-env with U87-CD4 cells, gray diamonds, n = 3; COS-env with sCD4, white diamonds, n = 2; COS-env with U87-CD4-CCR5 cells, each fixed separately prior to mixing for immunization, gray squares, n = 2), or mock-transfected cos cell immunogen (cocultured with U87-CD4-CCR5 cells, white circles, n = 2). Unimmunized mice (white triangles, n = 2) were also used. Neutralization was independent of specific co-receptor use and data here represent averages of three to six neutralization assays in U87-CD4-CXCR4 or -CCR5 cells. In some cases, sera from all animals within each experimental group were pooled. These results are consistent with the well-documented failure of rgp120 vaccines to elicit PI virus neutralization. The difference in neutralization by FC and FI vaccine sera was also observed in assays utilizing human primary blood lymphocytes (PBLs) (Fig 2B).

Neutralization of the homologous 168P PI virus in human PBL culture by FC, but not FI immunogens was also demonstrated. As shown in Fig 2B, lymphocytes were isolated, stimulated with phytohemagglutinin, and grown in the presence of interleukin-2; neutralization was determined as described herein. HIV p24 antigen was determined after 5 days of culture by ELISA (Coulter Corporation) and values were normalized to the virus control (36 ng/ml). * indicates p24 antigen levels below the limit of detection at the dilution

used in the ELISA. Vaccine groups were as defined in Fig 2A, and sera were pooled for this assay.

Specificity of the FRMS immunogen

In order to exclude the possibility that FC vaccine sera inhibited viral infectivity in a nonspecific manner, FC vaccine sera were shown not to inhibit infectivity of pseudotyped HIV virions bearing an amphotropic murine leukemia virus (MLV) envelope protein (Envelope-defective HIV NL4-3-Luc-R$^-$E$^-$ provirus was pseudotyped using amphotropic MLV envelope protein (Deng, H., et al., 1996, Nature 381:661). Further, FC vaccine sera were shown not to neutralize a primary isolate of the simian immunodeficiency virus SIVmac251. A primary isolate of SIVmac251 (Langlois, A.L., et al., 1998, Journal of Virology 72:6950) produced in rhesus PBLs was used (Fig 3).

As shown in Fig. 3, FC vaccine serum did not neutralize pseudotyped HIV virions bearing amphotropic MLV envelope protein or primary SIVmac251. HIV bearing an amphotropic MLV envelope protein (ampho MLV pseudotype) was used in neutralization sensitivity using pooled FC and FI antisera determined in U87-CD4-CXCR4 cells. Primary isolate SIVmac251 neutralization was determined in U87-CD4-CCR5 cells. Symbols are as defined in Fig 2 FC immunogen (black squares) and FI immunogen (Cos-env + U87 cells, black circles).

Laboratory-adapted Isolate Neutralization

As with conventional rgp120 vaccines, FI vaccines were able to elicit neutralization of a related laboratory-adapted isolate of HIV, the T-cell line adapted derivative of 168P, 168C (described in Wrin, T., et al., 1995, *Science* 69:39; LaCasse, R.A., et al., 1998, *Science* 72:2491). Neutralization sensitivity of the 168P PI virus and its TCLA derivative 168C were tested in U87-CD4-CXCR4 cells. Vaccine groups and symbols are as defined in Fig 2; sera were pooled for this assay. Results demonstrated in Fig 4, indicate neutralization of TCLA 168C virus by FI vaccine sera. Neutralization titers of the TCLA 168C virus were comparable among FC or FI vaccine sera, as were titers of anti-gp120 antibodies, suggesting a similar degree of inherent immunogenicity among the vaccines.

The failure of FI vaccines to elicit PI virus neutralization in the transgenic mouse model highlights the specificity of the neutralization elicited by FC vaccines. A statistical comparison was performed on the data set comprising all experimental animals and all virus neutralization assays. A simple model for virus-antibody binding was used to calculate a 'binding constant' K for each assay, and a mean K value was determined for each mouse. Analysis of variance with follow-up tests demonstrated a significant difference

in mean neutralization between FC and FI immunogens ($p < 0.01$). In all experiments, responses within experimental groups were consistent and uniform.

Furthermore, the consistent failure of FI vaccine sera to inhibit PI virus infectivity argues strongly that the immune response may not be directed to adventitious human cellular targets, such as those that confounded early studies of inactivated SIV vaccines (*see*, Cranage, M.P., et al., 1993, AIDS Research and Human Retroviruses 9:13; Putkonen, P., et al., 1993, Journal of Medical Primatology 22:100; Arthur, L.O., et al., 1992, Science 258:1935). Rather, the present invention recognizes that FC or FRMS immunogens present unique determinants that mediate neutralization of PI viruses.

Primary Isolate Neutralization by FRMS Vaccinated Sera

A critical issue in HIV vaccine development centers on the ability of vaccine antisera to neutralize a broad range of diverse PI viruses. In order to demonstrate the breadth of PI virus neutralization elicited by FRMS immunogens such as an FC immunogen, we examined the sensitivity of a panel of representative PI viruses from five prevalent and geographically-diverse phylogenetic clades. Infectious proviruses ACH320.2A.1.2 (320SI) and ACH320.2A.2.1 (320NSI) (Groenink, M., et al., 1991, Journal of Virology 65:1968; Guillon, C., et al., 1995, AIDS Research and Human Retroviruses 11:1537) were obtained through the NIBSC (UK) AIDS Reagent Program). HIV89.6 (Collman, R., et al., 1992, Journal of Virology 66:7517), SHIV89.6, and SHIV89.6P (Reimann, K.A., et al., 1996, Journal of Virology 70:3198) were also used. All other primary isolates were obtained through the NIH AIDS Research and Reference Reagent Program and the UNAIDS Network for HIV -1 Isolation and Characterization. PI viruses were subjected to limited expansion in PHA-activated PBLs (Wrin, T., et al., 1995, *Science* 69:39).

As depicted in Fig 5, FC sera elicited by a functioning clade B envelope protein were able to neutralize 23 of 24 PI viruses tested - monocytropic/NSI and T-lymphocytropic/SI viruses from North America/Europe (clade B), Africa (clades A and D), Thailand (clades B and E), and India (clade C). Vaccine groups and symbols are as defined in Fig 2; sera were pooled for this assay.

Despite the sequence diversity among these isolates, most were similarly sensitive to neutralization by FC vaccine sera. One isolate (92RW008) failed to attain >50% neutralization and two others (93IN904 and 92UG024) showed limited neutralization above 50%; these exceptions to the otherwise broad pattern of neutralization further argue that the FC immunogens of the invention may target primarily viral determinants.

FI sera were uniformly unable to neutralize these heterologous PI viruses. The broad and uniform neutralization of diverse PI viruses by the FRMS immunogens

suggests that the critical determinants presented by FRMS immunogens (such as FC immunogens) are highly conserved, and may be intimately tied to the basic functioning of the envelope protein in binding and fusion.

Neutralizing Antibodies

In order to more precisely define the molecular target for PI virus neutralization by FC vaccines, neutralizing antibodies were attempted to be removed from FC vaccine sera on incubation with envelope protein expressed on the surface of transfected COS cells.

Accordingly, formaldehyde-fixed COS cells expressing 168P envelope protein were incubated with FC serum and the recovered serum was then tested for PI virus neutralization. FC vaccine serum was sequentially adsorbed four times with approximately 10^6 formaldehyde-fixed COS cells expressing 168P envelope. Incubations were for 1 hr at 4° with rocking. Controls included prebleed serum and formaldehyde-fixed mock-transfected COS cells. Adsorption of bulk anti-gp120 antibody was monitored by gp120 ELISA. Final sera were tested for neutralization of HIV 168P using U87-CD4-CXCR4 cells. Neutralization activity in FC vaccine serum was removed by incubation with envelope-expressing cells, but only minimally reduced by incubation with COS cell controls (Fig 6 and Fig 7). Specifically, FC vaccine serum was repeatedly incubated with formaldehyde-fixed COS-env or control COS cells and tested for residual neutralization of 168P using U87-CD4-CXCR4 cells. Serum obtained prior to FC immunization was similarly adsorbed.

Production of Neutralizing Antibodies

In order to demonstrate that the methods of the invention are useful in generating neutralizing antibodies, hybridoma supernatants were made by the methods of the invention (using cross-linked COS-env + U87-CD4-CCR5 as an immunogen) were capable of neutralizing primary isolates. Briefly, hybridomas assayed for virus neutralization in the U87-CD4-CXCR4 cell assay as described above. Hybridoma supernatants were tested for the ability to neutralize two representative HIV clade B isolates (ACH168.10 (168P) and 92US657). Neutralization is expressed as the number of foci in the presence of hybridoma supernatant relative to the number in the presence of medium alone. Each point represents a selected hybridoma. As shown in Fig. 8, a majority of hybridomas were found to neutralize both PI viruses to 60-80% (0.4 - 0.2 fraction infectivity remaining, respectively).

Thus, the FRMS immunogens of the invention are capable of eliciting neutralizing monoclonal antibodies against primary isolate virus. Taken together, this

example illustrates that an appropriately-presented clade B envelope protein can elicit potent neutralization against most PI viruses from multiple HIV clades and suggest that broad vaccine protection may not require an unlimited number HIV serotypes.

Further, although the static forms of the envelope protein does not function as an effective immunogen, the present invention recognizes that the critical fusion-related epitopes are sufficiently represented on the static protein to allow binding. When used in vaccine formulations, the FRMS immunogens of the invention provide broad protection against a wide variety of viral serotypes and clades.

Example 2: Fusion-Defective Mutations

A variety of mutations have been examined for use in the methods of the invention.

In the case of HIV, three classes of mutations of the envelope protein include, but are not limited to: (1) mutations that abrogate proteolytic cleavage of the gp160 precursor protein; (2) mutations that affect the N-terminal gp41 fusion peptide domain; and (3) mutations that alter the coiled-coil domain.

Mutations that abrogated proteolytic cleavage of the gp160 precursor proteins include certain mutations that altered the highly conserved K/R-X-K/R-R site at the C-terminus of gp120 prevent proteolytic cleavage of the gp160 polyprotein and also abrogate fusogenicity (Freed, E.O., et al., 1989, J. Virol. 63:4670-5; Guo H.G., et al., 1990, Virology, 174:217-24; Bosch V., et al., 1990, J. Virol. 1990:2337-44; Dubay, J.W., et al., 1995, J. Virol., 69:4675-82).

Site-directed mutagenesis (QuikChange, Stratagene) was used to introduce a documented cleavage site mutation into the 168P envelope protein (REKR to REKT). The cleavage-defective 168P envelope protein gp160 was expressed on the cell surface but was unable to mediate cell-cell fusion or infection by pseudotyped HIV virions.

Mutations that affect the N-terminal gp41 fusion peptide domain include mutation of the hydrophobic N-terminal region of gp41 which region is believed to mediate fusion by inserting into the cell membrane and destablizing the lipid bilayer. Certain amino acids changes within this region render the envelope protein nonfusogenics synthetic peptides.

Mutations in the N-terminal fusion peptide of gp41 have been well characterized: V2E (Freed, E.O., et al., 1992, Proc. Natl. Acad. Sci. USA 1992:70-4; Pereira, F.B., et al., 1997, AIDS Res. & Hum Retrovirus 13:1203-11) and G10V (Delahunty, M.D., et al., 1996, Virology 218-94-102). The former involves a polar substitution in the hydrophobic peptide, whereas the latter may affect the helical structure assumed in the lipid bilayer. These mutations are introduced into the 168P envelope gene

(gp41: AVGIGVLFLGFLG..) by site-directed mutagenesis and the envelope proteins is tested for expression are fusogenicity. Incorporation of these mutations into pAbT4587-168Penv allows facile generation of the cognate recombination vaccinia viruses of the invention.

Mutations that alter the coiled-coil regions include mutations that are within the highly conserved coiled-coil motif is found in fusion proteins of many virus families (Weissenhorn, W., et al., 1998, Proc. Natl. Acad. Sci. USA 95:6032-6), for example, V570R and Y586E of HIV. In one embodiment, changes are introduced into the 168P envelope gene and tested for expression and fusogenicity in cell assays before being transferred to pAbT4587-168Penv for generation of the cognate recombinant vaccinia virus.

Example 3 -Preparation of FRMS

Fusion-competent immunogens were prepared by co-cultivating calcium phosphate-transfected COS-7 (American Type Culture Collection, Rockville, MD) cells expressing the molecularly cloned HIV 168P envelope protein (from ACH168.10 a syncytium-inducing primary isolate that utilized both CCR5 and CXCR4) (LaCasse, R.A., et al., 1998, Science 72:2491) with an equal number of cell fusion partners. The cell fusion partners were U87-CD4 cells expressing CCR5 (U87-CD4-CCR5) (Dan Littman, Skirball Institute of Biomolecular Medicine, NYU Medical Center). Cultures were terminated at the onset of cell-cell interaction (3-5 hr) by fixation with ice-cold 0.2% formaldehyde in phosphate-buffered saline. The harvest was timed towards capturing transitional intermediates leading to cell-cell fusion. Preliminary and contemporaneous immunostaining studies indicated 10-30% of maximal syncytium formation at the time of harvest. Cells were collected by scraping following overnight fixation.

Example 4-Immunization of mice with the FRMS

Transgenic mice expressing both human CD4 and CCR5 co-receptor (Dan Littman, Skirball Institute of Biomolecular Medicine, NYU Medical Center) were immunized three times at 21 day intervals with cells prepared and collected as in Example 1. Cells were formulated as vaccine using Ribi Adjuvant (Ribi ImmunoChem Research, Inc.) ($2X10^6$ cells/ 0.2 ml/mouse). Blood was collected from the tail vein. Sera obtained were used in neutralization assays in U87-CD4 cell lines expressing either CCR5 or CXCR4. This rapid PI virus neutralization assay has been validated relative to a standard neutralization assay in peripheral blood lymphocyte (PBL) culture (LaCasse, R.A., et al., 1998, *Science* 72:2491), and performs well in the presence of mouse serum. In three separate experiments, fusion-competent vaccines were administered to three mice. Virus neutralization assays using the homologous PI virus 168P were performed using both

U87-CD4-CCR5 and -CXCR4 cells, and in some cases sera were analyzed after the second as well as the third immunization. In all cases, results were concordant and are averaged in the data presented in Fig 9. Potent neutralization of the homologous 168P PI virus was consistently observed; neutralization levels of ≥ 80% were typically obtained at a 1:100 dilution of mouse serum. Neutralization was observed using CXCR4, a co-receptor to which the animal had not been exposed.

Several critical control immunizations were performed. One fundamental control was to confirm that non-functioning envelope protein immunogens did not elicit PI virus neutralizing antibodies in the transgenic mouse vaccination model. In one study, one mouse received vaccine comprising 168P envelope/COS cells that had been cocultured with U87 cells expressing neither CD4 or co-receptor. Although some neutralization of 168P PI virus was observed in a total of three assays from two bleeds, the titer and extent were quite distinct from that observed using fusion-competent vaccines.

Conformational changes induced upon CD4 binding are believed to be critical for subsequent interaction with co-receptor. Therefore, antibodies directed to these novel envelope proteins are advantageous for PI virus neutralization. In parallel with the envelope protein control vaccine, another mouse received control vaccine comprising COS cells expressing 168P envelope that had been cocultured with U87-CD4 cells. No syncytium formation was observed using this fusion-incompetent system. It appears that the addition of CD4 contributed little to the very minimal neutralization obtained using envelope protein alone.

Residual neutralization might arise from antibodies directed to CD4 and CCR5 were tolerance to the transgenes to be incomplete. Thus, control vaccines comprising U87-CD4-CCR5 cells, alone or cocultured with mock-transfected COS cell, were tested. In three studies, no inhibition of viral infectivity was observed. These data confirm tolerance in these transgenic mice and validate the vaccination model. Specific immune responses appear limited to viral and perhaps virus-induced epitopes. Not only do these mice appear tolerant to human CD4 and CCR5, as would human vaccinees, but antibodies directed to other cellular proteins do not appear to interfere with viral infectivity in this assay.

The possibility that the antibody response might be in part mediated by DNA immunization was explored. During transfection, envelope-expressing cells were exposed to microgram quantities of plasmid DNA. To test whether animals were responding to DNA immunization, two mice were injected subcutaneously with 20 μg 168P23 plasmid (in the pCR3.1-Uni expression vector). Although this route of administration is not preferred for DNA immunization, it does mirror that used with transfected cellular vaccines. The DNA

was not formalin-inactivated in this experiment. In a total of 8 virus neutralization assays, no PI virus neutralization was observed upon DNA immunization.

Example 5-Neutralization of Additional PI Using Antibodies to the FRMS

ACH320.2A.1.2 (320SI) (Amsterdam Cohort) is a molecularly cloned T-lymphocytropic PI virus which is particularly refractory to neutralization by HIV-IG, CD4-Ig, and IgG_1-b12. Residual sera remaining in Example 3 were used to test the sensitivity of this PI virus to fusion-competent and fusion-incompetent immunogens. Although limited to a starting 1:100 dilution of fusion-competent serum, this heterologous clade B isolate was clearly sensitive to neutralization. Of interest also was the failure of the fusion-incompetent sera (env and env + CD4) to neutralize the heterologous 320SI virus, in contrast to the partial neutralization seen against the homologous 168P virus. (Fig. 10)

A critical issue in HIV vaccine development centers on the ability of vaccine antisera to neutralize a broad range of diverse PI viruses. To determine the breadth of PI virus neutralization elicited by fusion-competent immunogens, the sensitivity of a panel of representative PI viruses was examined from five prevalent and geographically-diverse phylogenetic clades. Neutralization assays in U87-CD4-co-receptor cells are as described above and in Example 1. Fusion-competent sera elicited by a functioning clade B envelope protein were able to neutralize 23 of 24 PI viruses tested-monocytropic/NSI and T-lymphocytropic/SI viruses from North America/Europe (clade B), Africa (clades A and D), Thailand (clades B and E), and India (clade C). Despite the sequence diversity among these isolates, most were similarly sensitive to neutralization by fusion-competent vaccine sera. One isolate (92RW008) failed to attain >50% neutralization and two others (931N904 and 92UG024) showed limited neutralization above 50%. Control sera were uniformly unable to neutralize these heterologous PI viruses, in keeping with the historic failure of rgp120 immunogens. The broad and uniform neutralization of diverse PI viruses elicited by a single representative, but appropriately presented, clade B envelope protein suggests that the critical determinants presented by fusion-competent immunogens are highly conserved, and may be intimately tied to the basic functioning of the envelope protein in binding and fusion. These findings suggest that the number of different HIV neutralization serotypes needed for worldwide protection against HIV infection may be limited.

Example 6-Preparation and isolation of tagged FRMS

The FRMS of the subject invention were tagged using a commercially available system to facilitate their isolation and purification. The molecular engineering methods used to construct S-peptide tagged CD4, CCR5, CXCR4 and HIV envelope are parallel and will be described in detail only for CD4-Spep. Molecules were tagged with an

S-peptide at the C-terminal end of the CD4 molecule. The S peptide-encoding sequence (lys-glu-thr-ala-ala-ala-lys- phe-glu-arg-gln-his-met-asp-ser) was inserted between the cytoplasmic C-terminal isoleucine and termination codon (TGA) of the CD4 cDNA expression plasmid (CD4-Spep) using a synthetic oligonucleotide and high fidelity XL PCR (PE Applied Biosystems). Functional expression of the modified CD4 was confirmed by infection of COS-7 cells expressing CD4-Spep and the CXCR4 co-receptor. The C-terminally tagged CD4 was able to support infection by 168P virus comparable to native CD4. S-peptide tagged CD4 was also readily isolated using S-protein affinity chromatography (Novagen, Inc.). Using similar methods, we have constructed homologous S-peptide tagged CCR5 and CXCR4 co-receptor and HIV envelope molecules. All constructs were shown to be functional in binding and fusion. COS-7 cells were transfected with CXCR4 and either CD4-Spep or native CD4 (in pcDNA3.1 expression vectors), and 2 days subsequently labeled at the cell surface with biotin (EZ-Link Sulfo-NHS-LC-Biotin; Pierce Chemical Co.). Lysates were prepared in 0.5% Triton X-100 with protease inhibitors. CD4 molecules were purified either by 1) immunoprecipitation using MAb T4 (Coulter Corp., Hialeah, FL) and rabbit-α-mouse Ig antibody-loaded Protein A agarose, or 2) affinity purification using S-protein agarose. Proteins were released by boiling in sodium dodecyl sulfate (SDS) sample buffer, resolved by 10% polyacrylamide-SDS gel electrophoresis, and transferred to nitrocellulose. Biotinylated proteins were detected by avidin-horseradish peroxidase (Pierce) and nickel-enhanced DAB substrate. S-protein agarose resulted in the specific purification of the 62kD CD4-Spep with yield and purity which surpassed that of α-CD4-antibody mediated immunoprecipitation.

The time course of S-peptide isolation following formalin crosslinking was examined. COS-7 cells were transfected with CD4-Spep (± CXCR4) and were subsequently harvested in 0.5% Triton X-100 lysis buffer (control), or subjected to ice-cold formaldehyde fixation prior to lysis. CD4-Spep protein was purified by S-protein agarose, and analyzed by Western blot using S-protein HRP (Novagen, Inc.). Minimal loss of S-peptide binding (and/or recovery) was seen with 0.2% formaldehyde fixation for 1 hr; some recovery was lost with 2% formaldehyde in 1 hr and more at 3 hr. Intermediate reaction conditions (*e.g.* 0.2% formaldehyde for 1-2 hr) will allow for efficient isolation of effectively cross-linked complexes.

To identify specific CD4-Spep complexes, experiments were performed using formaldehyde fixation as well as the specific cross-linking agent DTSSP (Pierce Chemical Company). DTSSP is a homobifunctional N-hydroxysuccinimidyl ester that reacts, as formaldehyde, with primary amines. Because of its negative charge, DTSSP cannot penetrate or cross the cell membrane and thus, in contrast to formaldehyde, is expected to spare the cytoplasmic S-peptide tag. Also unlike formaldehyde, DTSSP

cross-linking is reversible; an internal disulfide linkage can be cleaved to release cross-linked components.

COS-7 cells were transfected with 168P envelope or CD4-Spep plasmids; envelope expressing cells as well as mock transfected cells were metabolically labeled in a 10 cm dish for 8 hr using 1 mCi total ^{35}S-methionine and cysteine. Envelope (or mock) and CD4-Spep expressing cells were then co-cultured for 4 hr to allow cell-cell interaction, and subjected to formaldehyde (0.2%, 1 hr on ice) or DTSSP (2 mM, 2X10 min on ice) cross-linking. Lysates were prepared and S-peptide containing complexes were isolated by S-protein agarose affinity chromatography.

Formaldehyde cross-linked complexes were analyzed by 6% polyacrylamide SDS gel electrophoresis. A high molecular weight complex that is not found in the mock + CD4-Spep control was identified. Viral proteins gp120 and gp160 were also visible, presumably isolated through non-covalent association with CD4-Spep. The detection of S-protein affinity-purified (*i.e.*, CD4-Spep) and metabolically-labeled (*i.e.*, envelope) high molecular weight complexes suggests that cross-linked complexes can be isolated for further study.

To continue the analysis of these high molecular weight complexes, DTSSP cross-linked complexes were examined. S-protein affinity purified complexes resolved by 6% polyacrylamide/SDS gel electrophoresis with or without prior treatment with 50 mM DTT were analyzed. Envelope-specific complexes were unable to be clearly resolved above those isolated from the mock + CD4-Spep control, with DTSSP cross-links intact. Little gp120/gp160 was detected. However, reversal of DTSSP cross-linking results in the specific release of gp120 and gp160 proteins. These data confirm that the formation of envelope-CD4 complexes observed using formaldehyde fixation, and highlight the power in the use of reversible cross-linking agents.

These data support the utility of the CD4-Spep affinity tag and the chemical cross-linking methodologies in the isolation of fusion-active CD4-associated complexes. S-peptide tagged co-receptors CCR5 (CCR5-Spep), CXCR4 (CXCR4-Spep), viral envelope (Env-Spep) have been produced and can also be used to isolate the fusion-active complexes. Isolated complexes can be used to define the molecular structure associated with the progression of envelope-mediated membrane fusion.

Studies were performed to determine whether envelope protein can be co-purified with either CD4-Spep or CCR5-Spep. Human 293T cells were separately transfected with 168P envelope protein, and a) CD4-Spep, or b) CCR5-Spep and CD4, or c) mock. Envelope-expressing cells were co-cultured with fusion partners (a-c) for 5 hr and complexes were solublized using 1% Brij-97 detergent (Lapham et al., 1996) and isolated using S-protein affinity chromatography. Co-purified envelope protein isolated using

Brij-97 was detected by Western blot analysis using the HIV V3-directed mAb 50.1 (Fig 11). As anticipated, a large amount of 168P envelope protein was isolable following co-culture with cells expressing CD4-Spep. A lesser, but significant, amount of envelope protein was also isolable following co-culture with cells expressing CD4 and CCR5-Spep. Contaminating envelope protein was not detectable from co-cultures with mock-transfected cells. Complexes were isolated from formaldehyde cross-linked fusion-competent co-cultures in similar experiments.

These results strongly suggest that complexes of envelope protein, CD4, and CCR5 arise early during fusion and can be isolated by S-protein affinity chromatography. These S-protein purified molecules and complexes can be used as "split virus" or subunit vaccine immunogens.

Example 7: Recombinant Vaccinia Viruses That Generate Fusion-Competent FRMS in Cell Culture or at the Site of Vaccination.

Other acceptable vaccine approaches to translate our invention into a practical formulation include the use of viral vectors. For instance, coadministration of recombinant vaccinia viruses that express env and CD4/CCR5, respectively, might drive cell-cell fusion *in situ*. In one embodiment, two recombinant vaccinia viruses-one expressing HIV 168P envelope protein (rV-168Penv) and the second expressing CD4 and CCR5 co-receptor (rV-CD4/CCR5) were constructed. Co-cultures of cells infected with rV-168Penv and cells infected with rV-CD4/CCR5 were fusion-competent and yield multinucleate syncytia (Fig. 12). rV-168Penv cells, rV-CD4/CCR5 cells, or cross-linked cocultured rV-168Penv and rV-CD4/CCR5 cells were used as FRMS to vaccinate transgenic mice, as described above.

Sera obtained from mice are vaccinated with cross-linked cocultured rV-168Penv and rV-CD4/CCR5 cells are capable of virus neutralization across a variety of clades of HIV indicating that the viral vectors may successfully be used in the methods of the invention in the formation of the FRMS.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modification or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing

description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein above, including patent applications, patents, and publications, the disclosures of which are hereby incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. An isolated molecular structure comprising an epitope formed as a result of association of (a) an envelope protein of an enveloped virus, with (b) one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins.

2. An isolated molecular structure comprising an epitope formed as a result of association of (a) an HIV envelope protein, or a mutant thereof that assembles into the viral envelope; with (b) human CD4 and a co-receptor for HIV fusion.

3. The molecular structure of claim 2 wherein the co-receptor is CCR5 or CXCR4.

4. The molecular structure of claim 2 which is formed by association of a mutant of HIV gp41 that is fusion-defective.

5. The molecular structure of claim 4 wherein the mutant contains one or more mutations selected from the group consisting of V2E, G10V, V570R, and Y586E.

6. The molecular structure of claim 2 which is formed by association of wild-type HIV envelope protein.

7. An isolated molecular structure comprising an epitope formed as a result of association of (a) a mutant envelope protein of an enveloped virus, which envelope protein in wild-type form functions in fusion of the viral envelope with a host cell membrane, and which mutant envelope protein is fusion-defective; and (b) one or more host cellular membrane proteins which function as receptors for said envelope protein.

8. The molecular structure of claim 1 or 7 wherein said virus is from a viral family is selected from the group consisting of Retroviridae, Rhabdoviridae, Caronaviridae, Filoviridae, Poxviridae, Bunyaviridae, Flaviviridae, Togaviridae, Orthomyxoviridae, Paramyxoviridae, and Herpesviridae.

9. The molecular structure of claim 1 or 7 wherein te envelope protein is E1 and E2 of HCV and the cellular membrane protein is CD81.

10. The molecular structure of claim 1 or 7 which is a cross-linked cellular molecular structure.

11. The molecular structure of claim 2 which is a cross-linked cellular molecular structure.

12. The molecular structure of claim 1 or 7 which is isolated from a cell lysate.

13. The molecular structure of claim 2 which is isolated from a cell lysate.

14. The molecular structure of claim 10 wherein the cellular molecular structure comprises cells recombinantly expressing the envelope protein.

15. The molecular structure of claim 11 wherein the cellular molecular structure comprises cells recombinantly expressing the envelope protein.

16. The molecular structure of claim 12 wherein the cell lysate is from a plurality of cells comprising cells recombinantly expressing the envelope protein.

17. The molecular structure of claim 14 wherein the cellular molecular structure further comprises cells recombinantly expressing the one or more host cellular membrane proteins.

18. A recombinant enveloped virus, wherein said virus recombinantly expresses on its envelope a cell receptor for a native envelope protein of said virus.

19. The virus according to claim 18 which is HIV, and wherein said cell receptor is human CD4 or a co-receptor for HIV, or said virus recombinantly expresses both human CD4 and said co-receptor.

20. The molecular structure of claim 1 wherein said suitable conditions comprise a lowering of pH.

21. The molecular structure of claim 11 wherein the cross-linked cellular molecular structure further comprises a cross-linked viral particle of said virus, containing said envelope protein.

22. A vaccine formulation comprising an immunogenic amount of the molecular structure of any one of claims 1-4, 6 and 8; and a pharmaceutically acceptable carrier.

23. A monoclonal antibody to the molecular structure of claim 1 or 7.

24. A monoclonal antibody to the molecular structure of claim 2.

25. Purified polyclonal antiserum specific to the molecular structure of claim 2.

26. A contraceptive jelly, foam, cream, or ointment comprising an amount of the antibody of claim 23 effective to inhibit or decrease infection by the virus.

27. A contraceptive jelly, foam, cream, or ointment comprising an amount of the antibody of claim 24 effective to inhibit or decrease infection by HIV.

28. A contraceptive jelly, foam, cream, or ointment comprising an amount of the antiserum of claim 25 effective to inhibit or decrease infection by HIV.

29. A sample of mammalian blood, to which an amount of the antibody of claim 23 has been added effective to inhibit or decrease infection by the virus.

30. A sample of human blood, to which an amount of the antibody of claim 24 has been added effective to inhibit or decrease infection by HIV.

31. The molecular structure of claim 1 or 7 wherein said envelope protein or host cellular membrane proteins further comprises an affinity tag.

32. The molecular structure of claim 2 wherein said envelope protein, CD4, or co-receptor further comprises an affinity tag.

33. The antibody of claim 23 which is labeled.

34. A kit comprising in one or more containers a labeled monoclonal antibody to the molecular structure of claim 1.

35. A kit comprising in one or more containers a labeled monoclonal antibody to the molecular structure of claim 2.

36. The kit of claim 34 which further comprises in a separate container the molecular structure of claim 1.

37. The kit of claim 35 which further comprises in a separate container the molecular structure of claim 2.

38. A cell line that recombinantly expresses an envelope protein of an enveloped virus that functions in fusion of the viral envelope with a host cell membrane, or a mutant form of said envelope protein that is fusion-defective, which cell line expresses one or more cellular membrane proteins that function as receptors for said envelope protein.

39. The cell line of claim 38 wherein said one or more proteins that function as receptors are recombinantly expressed.

40. A cell line that recombinantly expresses HIV gp160, which cell line expresses CD4 and a co-receptor for HIV; said cell line lacking a functional protease that cleaves gp160 to produce gp120 and gp41.

41. A method of treating or preventing infection by a virus in a subject comprising administering to the subject an immunogenic amount of the molecular structure of claim 1 or 7 effective to treat or prevent infection by the virus.

42. A method of treating or preventing infection by a virus in a subject comprising administering to the subject an immunogenic amount of the molecular structure of claim 10 effective to treat or prevent infection by the virus.

43. A method of treating or preventing infection by a virus in a subject comprising administering to the subject an immunogenic amount of the molecular structure of claim 12 effective to treat or prevent infection by the virus.

44. A method of treating or preventing infection by HIV in a human comprising administering to the human an immunogenic amount of the molecular structure of claim 2 effective to treat or prevent infection by HIV.

45. A method of treating or preventing infection by HIV in a human comprising administering to the human an immunogenic amount of the molecular structure of any of one claims 3-6 effective to treat or prevent infection by HIV.

46. The method of claim 41 wherein the subject is a human.

47. The method of claim 41 wherein the subject is a domestic animal.

48. A method of treating or preventing infection by a virus in a subject comprising administering to the subject an amount of the monoclonal antibody of claim 23 effective to treat or prevent infection by the virus.

49. A method of treating or preventing infection by HIV in a human comprising administering to the human an amount of the monoclonal antibody of claim 24 effective to treat or prevent infection by HIV.

50. The method of claim 49 wherein said human has a high risk of HIV infection.

51. A method for treating or preventing infection by HIV in a human fetus comprising administering to a pregnant human containing said fetus an amount of the monoclonal antibody of claim 24 effective to treat or prevent infection by HIV in said fetus.

52. The method of claim 49 which is for treatment of AIDS in said human.

53. A method of inhibiting infection by a virus in a sample of blood comprising contacting said sample of blood with an amount of the monoclonal antibody of claim 23 effective to inhibit infection by said virus.

54. A method of inhibiting infection by HIV in a sample of human blood comprising contacting said sample of human blood with an amount of the monoclonal antibody of claim 24 effective to inhibit infection by HIV.

55. A method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody of claim 23 effective to inhibit infection by said virus.

56. A method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody of claim 24 effective to inhibit infection by HIV.

57. A method for monitoring the production of antibody to the molecular structure of claim 1 or 7 in a subject previously administered an amount of the molecular structure of claim 1 or 7, comprising isolating from said subject a sample comprising serum; and detecting the presence of any antibodies to the molecular structure of claim 1 or 7 in said serum.

58. The method of claim 57 wherein said detecting is carried out by a method comprising performing a competitive immunoassay with labeled antibody to the molecular structure of claim 1 or 7.

59. A method of producing an immunogen for use in a vaccine for the treatment or prevention of infection by a virus comprising the following steps in the order stated:

(a) contacting an envelope protein or chimeric form thereof of an enveloped virus, which envelope protein functions in fusion of the viral envelope with a cell membrane, or a mutant form or chimeric form thereof of said envelope protein that is fusion-defective, with one or more cell proteins or chimeric forms thereof that function as receptors for said envelope protein; and

(b) exposing said envelope protein or chimeric form thereof or mutant form or chimeric form thereof, and said one or more host cell proteins or chimeric forms thereof, to a cross-linking agent; and

(c) isolating a cross-linked structure comprising said envelope protein or chimeric form thereof or mutant form or chimeric form thereof.

60. The method of claim 59 wherein said virus is HIV and said host cell proteins are human CD4 and a co-receptor for HIV.

61. A method of producing an immunogen for use in a vaccine for the treatment or prevention of infection by HIV comprising the following steps in the order stated:

(a) co-culturing a first cell recombinantly expressing HIV envelope protein or a chimeric form thereof, or a mutant form or chimeric form thereof of said envelope protein that is fusion-defective, with a second cell that expresses (i) human CD4 or a chimeric form thereof, and (ii) a co-receptor for HIV or a chimeric form thereof; and

(b) exposing said envelope protein or chimeric form thereof or mutant form or chimeric form thereof, and said CD4 or chimeric form thereof and co-receptor or chimeric form thereof, to a cross-linking agent; and

(c) isolating a cross-linked structure comprising said envelope protein or chimeric form thereof or mutant form or chimeric form thereof.

62. The method of claim 61 wherein said second cell recombinantly expresses CD4 or said co-receptor or both CD4 and said co-receptor or chimeric forms of any of the foregoing.

63. The method of claim 61 wherein said first and second cell are the same cell type.

64. The method of claim 61 wherein said first and second cell are different cell types.

65. The method of claim 59 wherein in step (a), said envelope protein or chimeric form thereof or mutant form or chimeric form thereof is present on a viral particle or virus-like particle.

66. The method of claim 61 wherein said cross-linked structure is a cross-linked cellular complex.

67. The method of claim 59 wherein the virus is selected from the group consisting of Retroviridae, Rhabdoviridae, Caronaviridae, Filoviridae, Poxviridae, Bunyaviridae, Flaviviridae, Togaviridae, Orthomyxoviridae, Paramyxoviridae, and Herpesviridae.

68. The method of claim 65 wherein said contacting step occurs by infecting cells expressing said host cell proteins with said virus expressing said envelope protein or chimeric form thereof or mutant form or chimeric form thereof.

69. The method of claim 59 wherein a chimeric form of said envelope protein or one of said host cell proteins is contacted, said chimeric form comprising an affinity tag.

70. The method of claim 61 wherein a chimeric form of said envelope protein or CD4 or said co-receptor is contacted, said chimeric form comprising an affinity tag.

71. A method of producing an immunogen for use in a vaccine for the treatment or prevention of infection by HIV comprising the following steps in the order stated:

(a) co-culturing a first cell recombinantly expressing HIV envelope protein or a chimeric form thereof, or a mutant form or chimeric form thereof of said envelope protein that is fusion-defective, with a second cell that expresses (i) human CD4 or a chimeric form thereof, and (ii) a co-receptor for HIV or a chimeric form thereof wherein at least one of said chimeric forms comprising an affinity tag is expressed; and

(b) lysing said co-cultured cells to form a cell lysate under non-denaturing conditions; and

(c) isolating from said cell lysate a molecular structure comprising said envelope protein or chimeric form thereof or mutant form or chimeric form thereof by a method comprising contacting said cell lysate with a binding partner to said affinity tag and recovering a molecular structure bound to said affinity tag.

72. A cross-linked structure that is the product of the method of claim 59.

73. A cross-linked structure that is the product of the method of claim 60.

74. A cross-linked structure that is the product of the method of claim 61.

75. A cross-linked structure that is the product of the method of claim 62.

76. A monoclonal antibody to the structure of claim 60 that neutralizes *in vitro* the following primary isolates of HIV: 92US657, 92US660, 92RW023, 93IN101, 92UG035, and 92TH023.

77. A contraceptive, jelly, foam, cream or ointment comprising an amount of the antibody of claim 76 to inhibit or decrease infection by HIV.

78. A method of treating or preventing infection by a virus in a subject comprising administering to the subject an immunogenic amount of the structure of claim 72 effective to treat or prevent infection by the virus.

79. A method of treating or preventing infection by HIV in a human comprising administering to the human an immunogenic amount of the molecular structure of claim 73 effective to treat or prevent infection by HIV.

80. A method of treating or preventing infection by HIV in a human comprising administering to the human an immunogenic amount of the molecular structure of claim 74 effective to treat or prevent infection by HIV.

81. A method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody of claim 76 effective to inhibit infection by HIV.

82. A method of decontaminating surgical or dental tools comprising contacting said tools with an amount of the monoclonal antibody of claim 23 effective to inhibit infection by said virus.

83. A method of screening a molecular structure for vaccine efficacy comprising immunizing a transgenic non-human mammal with the molecular structure of claim 2, wherein said transgenic non-human mammal expresses from one or more transgenes both human CD4 and a co-receptor for HIV, and detecting any neutralizing antibodies to HIV that are produced by said mammal.

84. A method of screening a molecular structure for vaccine efficacy comprising immunizing a transgenic non-human mammal with the molecular structure of claim 1, wherein said transgenic non-human mammal expresses from one or more

transgenes said one or more host cellular membrane proteins; and detecting any neutralizing antibodies to said virus that are produced by said mammal.

85. The mammal of claim 83 which is a mouse.

86. The method of claim 61 wherein said first cell recombinantly expresses HIV envelope protein or a chimeric form thereof.

87. A kit comprising in one or more containers, the molecular structure of claim 1.

88. The kit of claim 87 which further comprises a pharmaceutically acceptable carrier, and wherein said molecular structure is present in an immunological amount.

89. An isolated protein complex comprising an envelope protein of human immunodeficiency virus type 1 functionally interacting with human CD4 and human CCR5.

90. A vaccine formulation comprising the protein complex of claim 89 and a pharmaceutically acceptable carrier.

91. A method of immunizing an animal to a virus comprising the steps of administering to the animal a vaccine formulation of claim 90 including a protein complex comprising one or more viral proteins functionally interacting with one or more host cellular receptors or co-receptors to mediate viral binding, entry and/or infection; whereby neutralizing antibodies to the virus is generated.

92. A method of preparing a protein complex comprising one or more viral proteins functionally interacting with one or more host cellular receptors or co-receptors to mediate viral binding, entry and/or infection, including the steps of:

a) culturing a first cell expressing one or more viral proteins;

b) culturing a second cell expressing one or more host cellular receptors or co-receptors for said one or more viral proteins'

c) co-culturing the first and second cells;

d) fixing said co-culture during cell-cell fusion; and

e) isolating the fixed cells.

93. Fixed cells made by the method of claim 92.

94. A method of purifying a protein complex comprising an envelope protein of human immunodeficiency virus type 1 functionally interacting with human CD4 and human CCR5 including the steps of:

a) tagging the complex with a peptide sequence to facilitate subsequent purification; and

b) isolating the tagged complex.

95. A method of treating or preventing infection by a virus in a subject comprising administering to the subject (a) a first nucleic acid encoding an envelope protein of an enveloped virus; and (b) a second nucleic acid encoding one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins, such that the envelope protein and cellular membrane proteins are expressed in the subject and neutralizing antibodies to the virus are produced.

96. The method of claim 95 wherein the first and second nucleic acids are the same.

97. The method of claim 95 wherein the first and second nucleic acid are different nucleic acid vectors.

98. The method of claim 95 wherein the envelope protein is en envelope protein of HIV, and the cellular membrane proteins are CD4 and an HIV co-receptor.

99. A vaccine formulation comprising (a) a first nucleic acid encoding an envelope protein of an enveloped virus; and (b) a second nucleic acid encoding one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins, such that the envelope protein and cellular membrane proteins are expressed in the subject and neutralizing antibodies to the virus are produced; and (c) a pharmaceutically acceptable carrier.

100. A method of treating a host that has been exposed to a virus, or preventing infection of a host by said virus, the method comprising the steps of administering to the host antibodies generated by immunizing an animal with the protein complex of claim 89 in an amount effective to treat or prevent infection of said host.

101. An isolated protein complex comprising an envelope protein of human immunodeficiency virus type 1 functionally interacting with human CD4 and human CCR5, wherein one or more of said envelope protein, CD4 or CCR5 is tagged with peptide.

102. A kit comprising in one or more containers (a) a first nucleic acid encoding an envelope protein of an enveloped virus; and (b) a second nucleic acid encoding one or more cellular membrane proteins, which envelope protein and cellular membrane proteins are necessary and sufficient under suitable conditions for fusion of said envelope of the virus with a cell membrane containing said cellular membrane proteins, such that the envelope protein and cellular membrane proteins are expressed in the subject and neutralizing antibodies to the virus are produced.

103. A composition comprising (a) the monoclonal antibody of claim 23; (b) said envelope protein of an enveloped virus; and (c) a pharmaceutically acceptable carrier.

104. A method of treating or preventing infection by a virus in a subject comprising administering to the subject an amount of the composition of claim 103 effective to treat or prevent infection by the virus.

105. The method of claim 92, wherein a vaccinia viral vector is used to express said one or more host cellular receptors or coreceptor in said second cell.

106. The method of claim 92, wherein a vaccinia viral vector is used to express said one or more viral protein in said first cell.

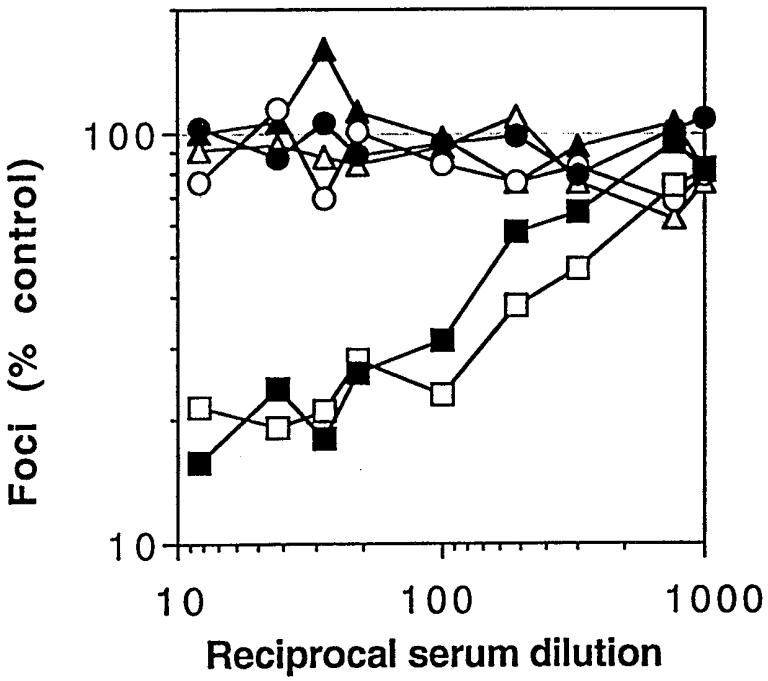
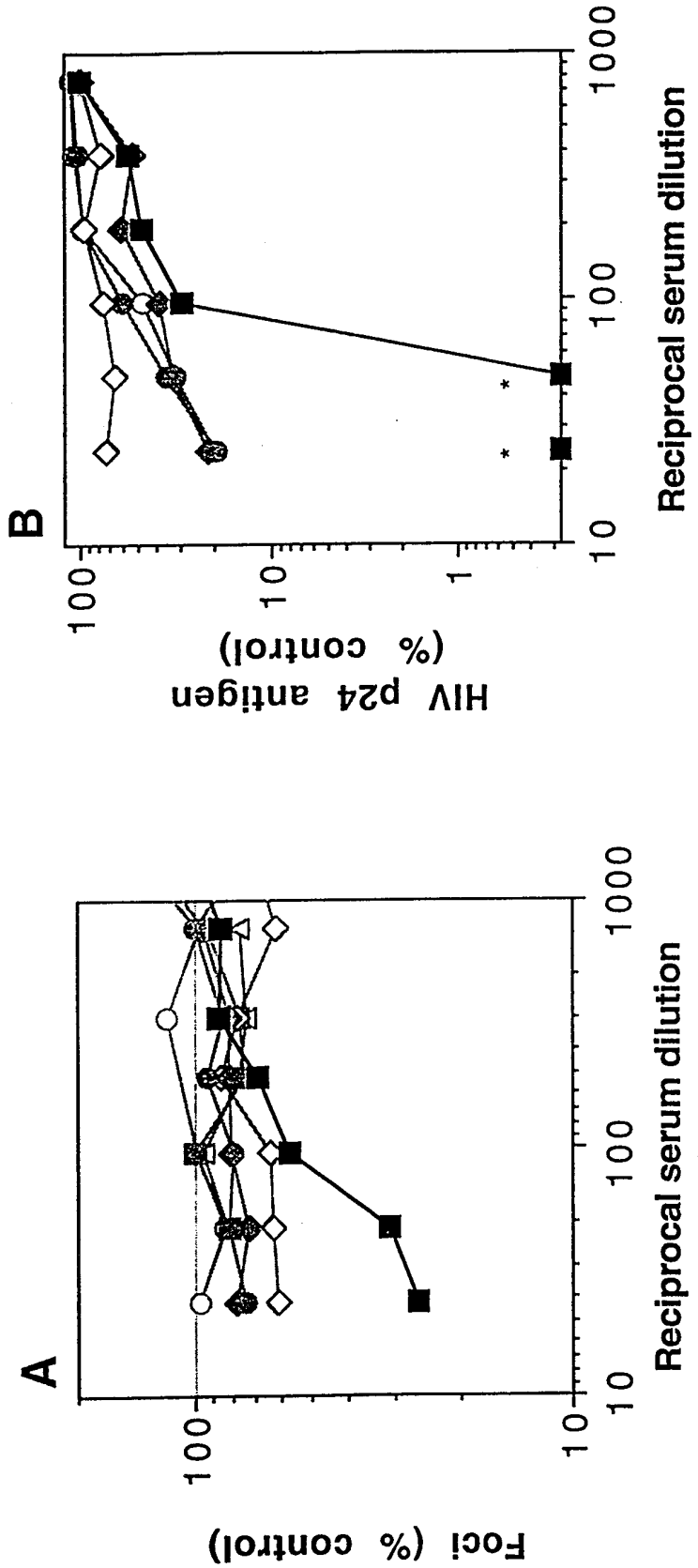
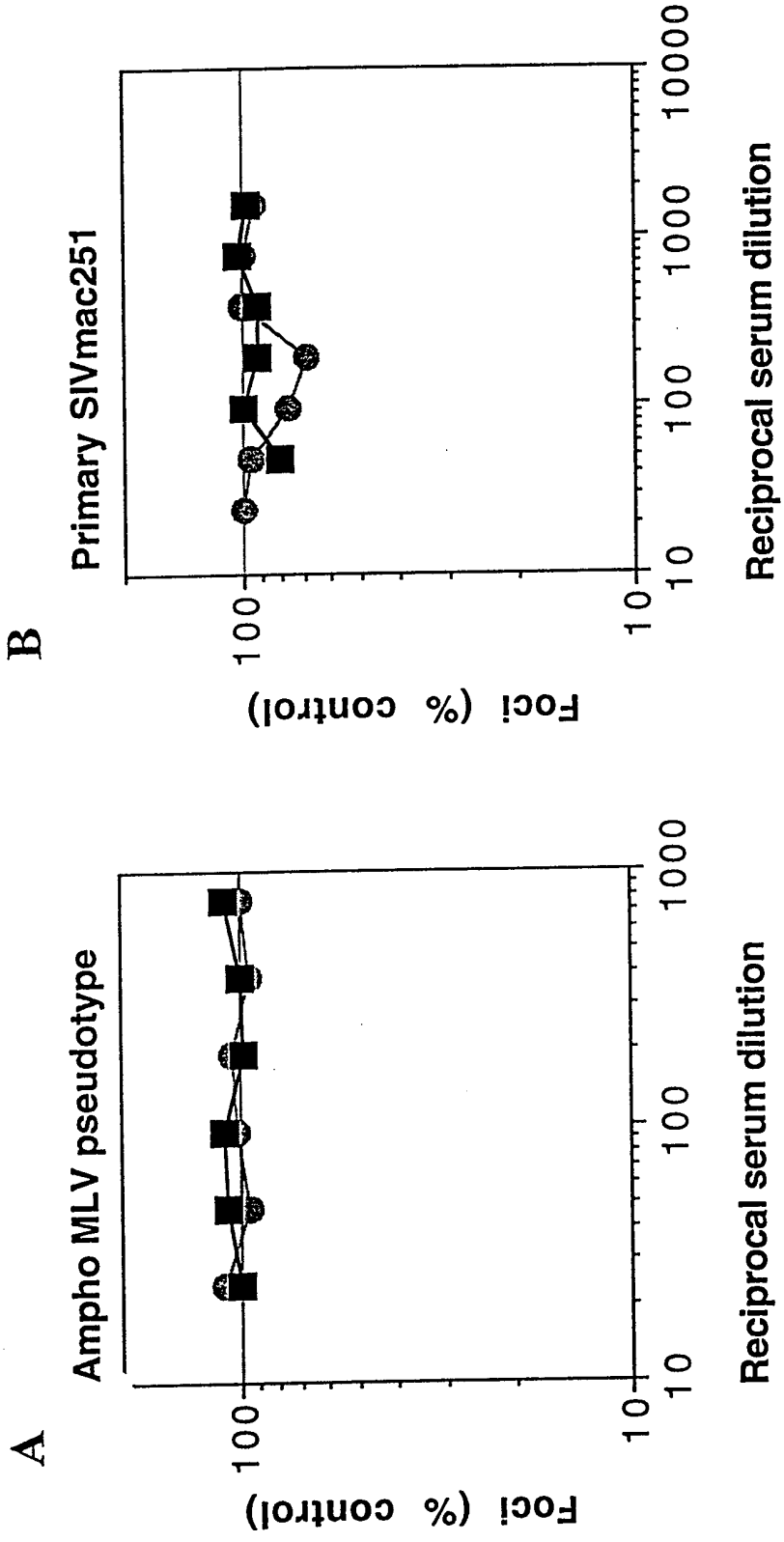


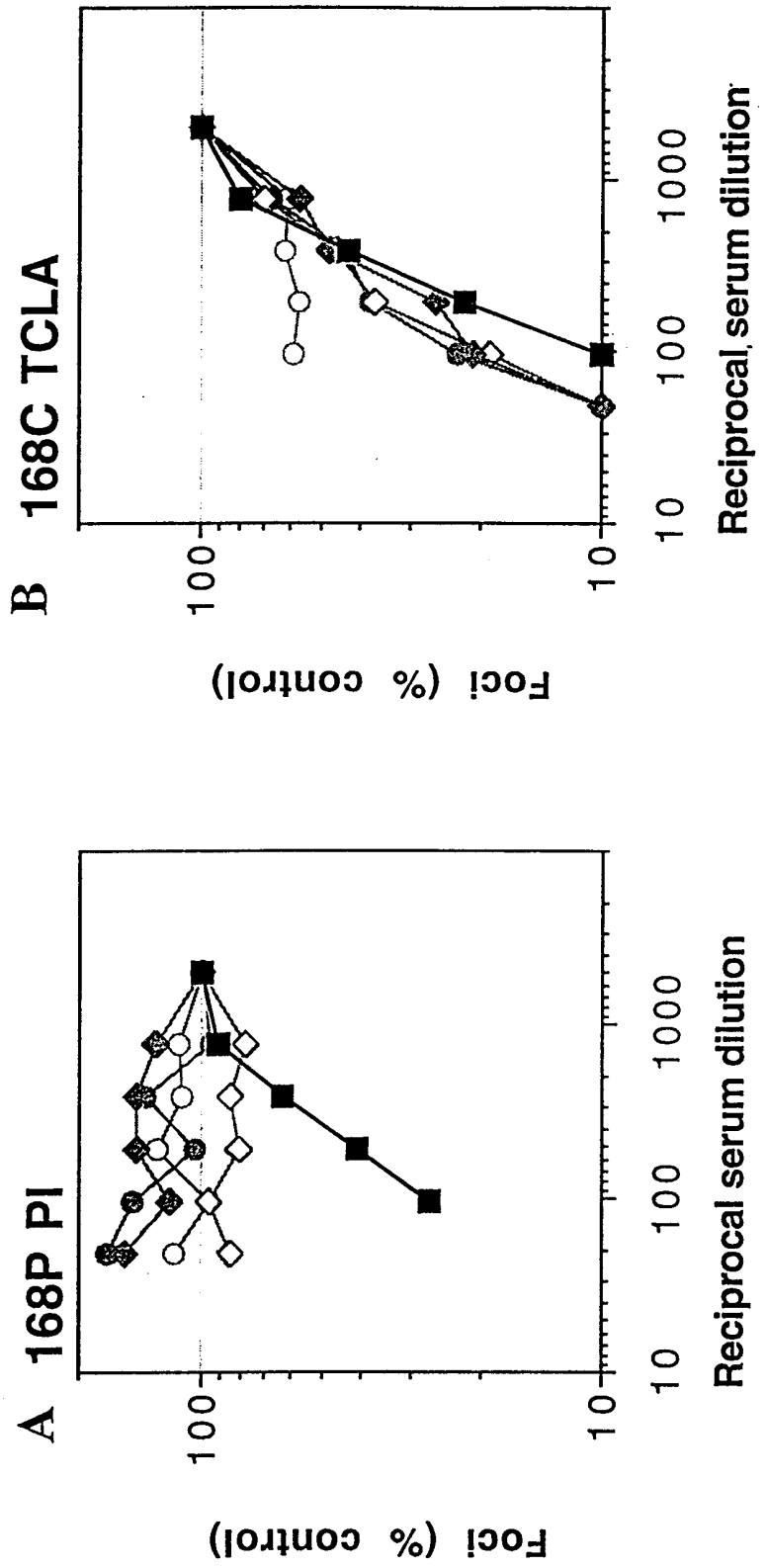
FIG. 1


B
HIV p24 antigen (% control)
100
10
1
10
100
1000
Reciprocal serum dilution
A
Foci (% control)
100
10
10
100
1000
Reciprocal serum dilution

FIGS. 2A-B


A
Ampho MLV pseudotype
Foci (% control)
100
10
10
100
1000
Reciprocal serum dilution
B
Primary SIVmac251
Foci (% control)
100
10
10
100
1000
10000
Reciprocal serum dilution

FIGS. 3A-B


B 168C TCLA
Foci (% control)
100
10
10
100
1000
Reciprocal serum dilution
A 168P PI
Foci (% control)
100
10
10
100
1000
Reciprocal serum dilution

FIGS. 4A-B

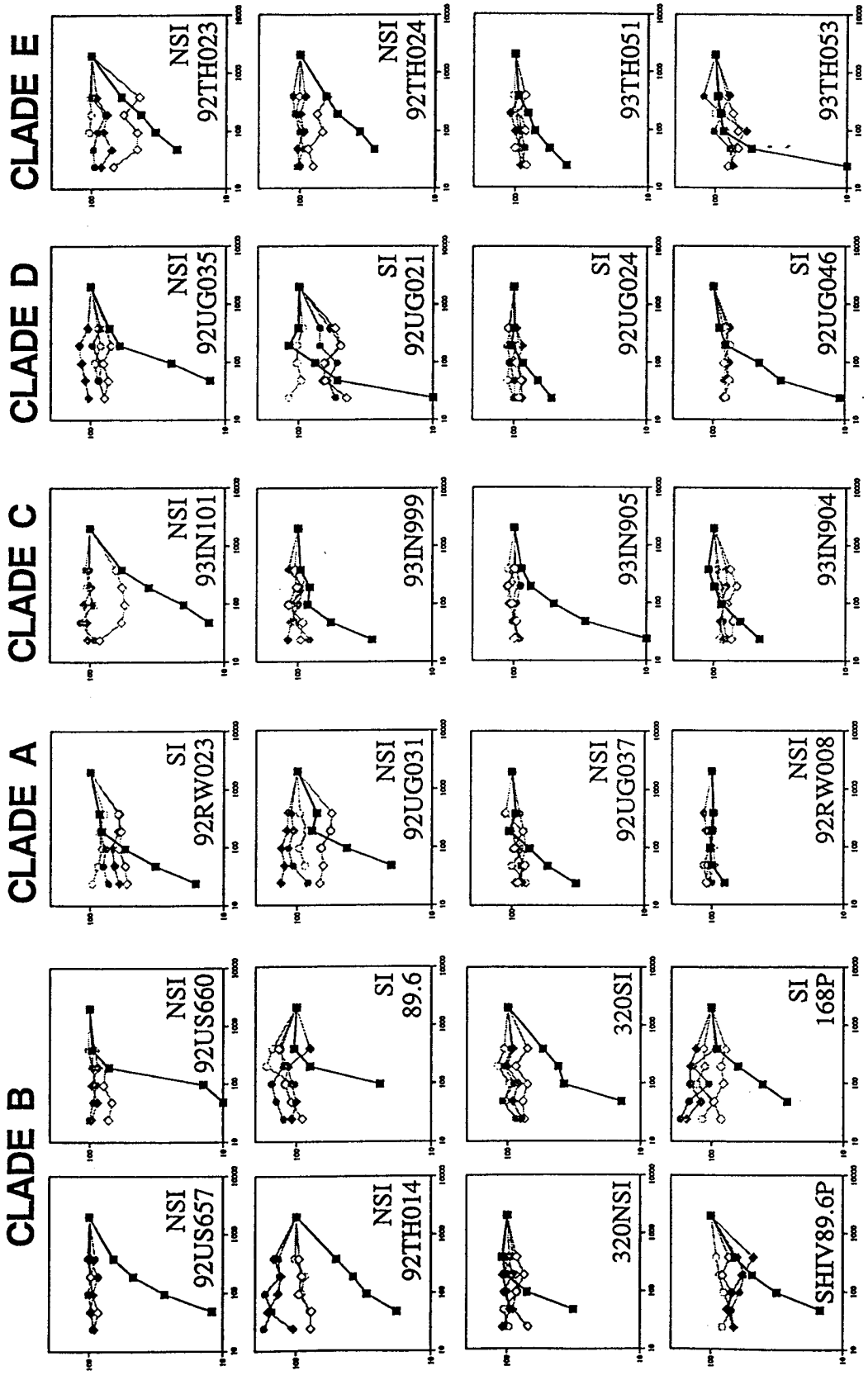

CLADE B
CLADE A
CLADE C
CLADE D
CLADE E
NSI 92US657
SI 92RW023
NSI 93IN101
NSI 92UG035
NSI 92TH023
NSI 92TH014
NSI 92UG031
93IN999
SI 92UG021
NSI 92TH024
320NSI
NSI 92UG037
93IN905
SI 92UG024
93TH051
SHIV89.6P
NSI 92RW008
93IN904
SI 92UG046
93TH053
Foci (% control)
Reciprocal serum dilution

FIG. 5

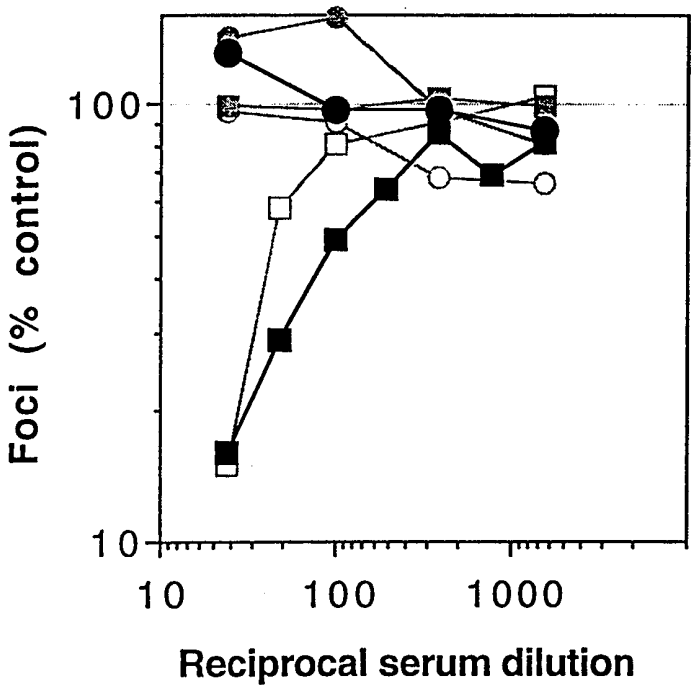


FIG. 6

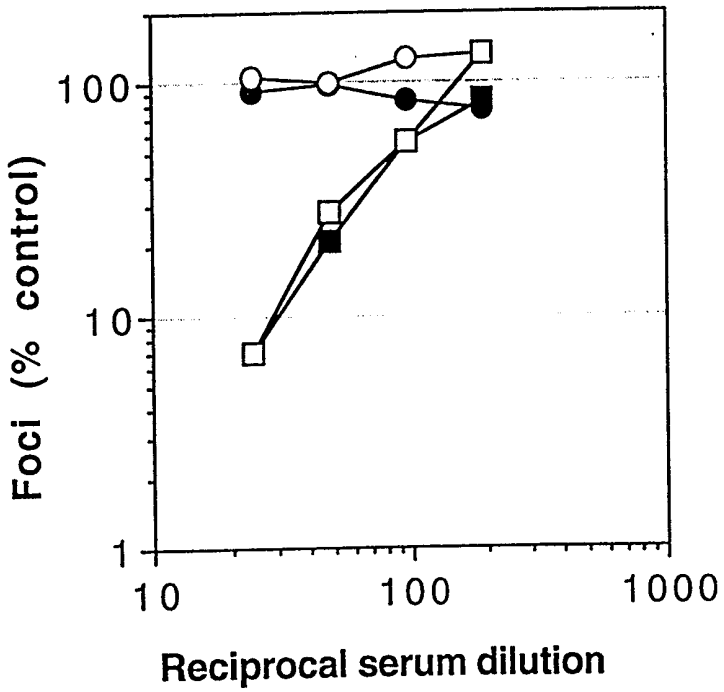


FIG. 7

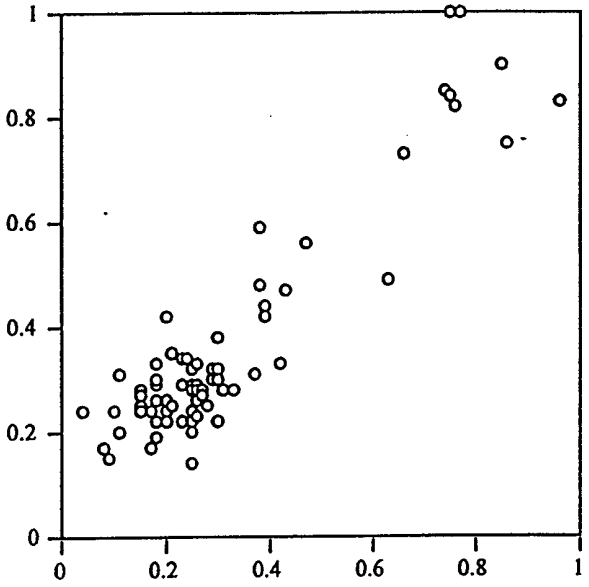

1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
Fraction infectivity remaining
(HIV ACH168.10)

FIG. 8

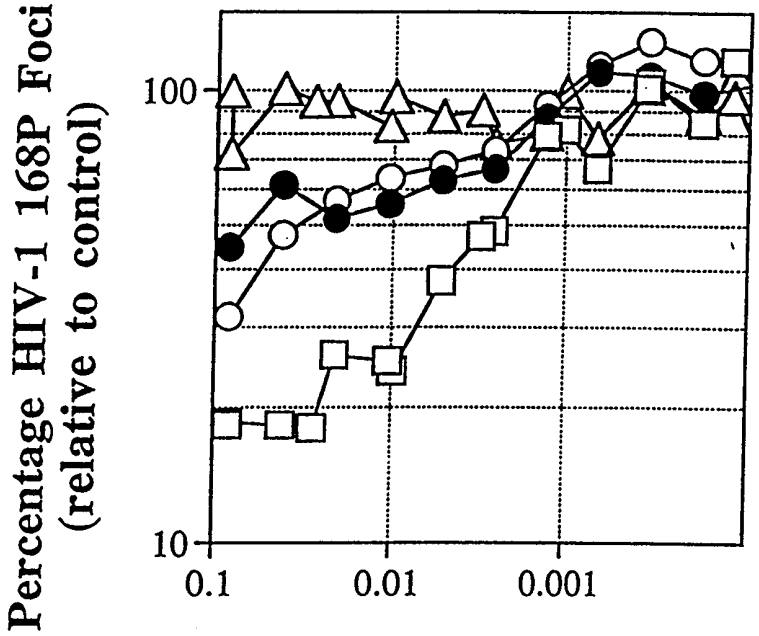

Percentage HIV-1 168P Foci
(relative to control)
100
10
0.1
0.01
0.001
Dilution Tg mouse serum
(hu CD4+, hu CCR5+, mu CD4+)

FIG. 9

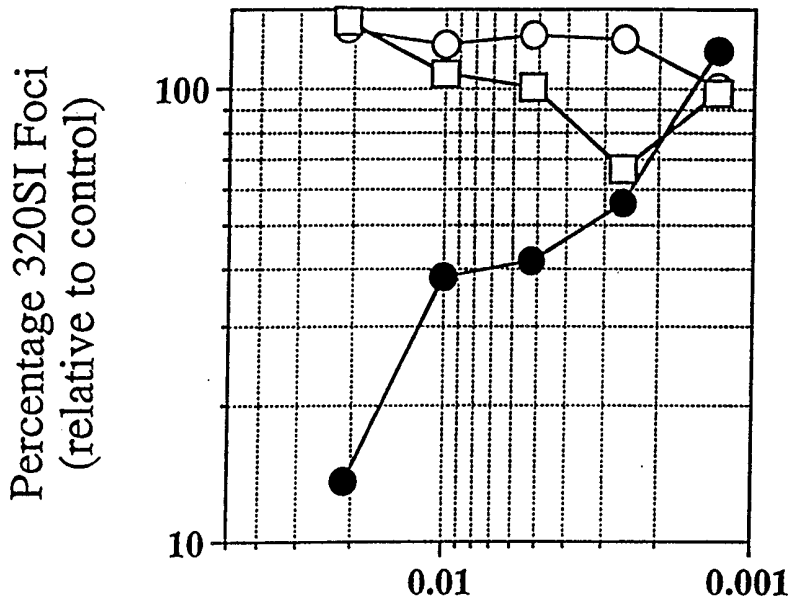

Percentage 320SI Foci
(relative to control)
100
10
0.01
0.001
Dilution Tg mouse Serum

FIG. 10

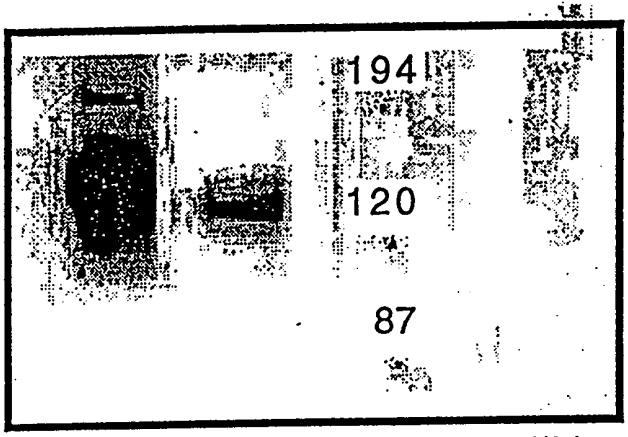


Fig 3: Co-purification of HIV envelope protein with CD4-Spep or CCR5-Spep.

FIG. 11

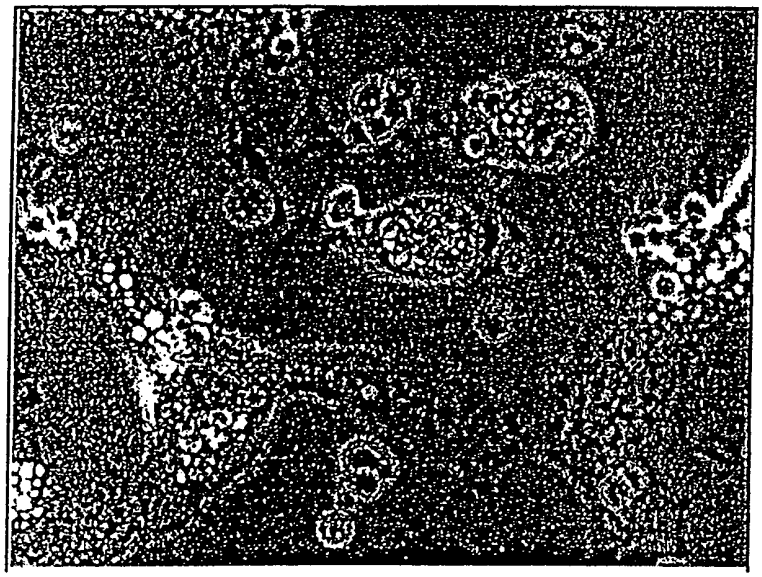


FIG. 12