**Title:** VIRUS-LIKE PARTICLES PRESENTING HIV-1 ENVELOPES, AND METHODS FOR MUCOSAL AND SUBLINGUAL IMMUNIZATION AGAINST HIV-1 USING THE SAME

**Abstract:** The present invention is directed to a method of expressing the human immunodeficiency virus (HIV-1) capsid protein coding sequence in a cell using an expression system under conditions facilitating expression of the protein in the cell. In another aspect of the invention, it has been designed a modified HIV-1 gp40 envelope protein made of the HIV-1 gp20 and the ectodomain of the gp41 linked at the NH2 with a signal sequence of the Honeybee Mellitein (HBMS) and at the COOH with the trans-membrane region (TM) of the baculovirus gp64 (HBMS-SOSIP gp40 gp64). In one embodiment of the invention, there is provided a method of expressing the gag major capsid protein of HIV-1 and the HBMS-SOSIP gp40 gp64 in several expression systems, including Si9 in insect cells using the baculovirus expression system, to obtain HIV virus-like particles (HBMS-SOSIP gp40 gp64 VLPs). It was further discovered that the HBMS-SOSIP gp40 gp64 VLPs show antigenic characteristics similar to those of native infectious HIV-1 particles and are recognized by sera from HIV-1 seropositive individuals. In yet another embodiment, the invention provides a method of vaccinating a mammal for HIV-1 by administering HIV-1 virus-like particles mucosally (including sublingual) to a mammal in an amount sufficient to induce an immune response to the HIV-1.
DESCRIPTION

FIELD OF THE INVENTION

The present invention relates generally to HIV-I. More particularly, the invention relates to a method of 1) expressing the human HIV-I major capsid protein coding sequence using several expression systems, including the eukaryotic baculovirus and the vegetal expression systems; 2) designing a modified HIV-I gpl40 envelope linked to heterologous signal sequence and trans-membrane regions; 3) production of HIV-I virus-like particles expressing on the surface a modified HIV-I gpl40 (HBM-SOSIPgpl40-gp64TM-VLPs) and use of these VLPs for mucosal (including sublingual) immunization of mammals to induce systemic and mucosal (Vaginal and rectal) immune response specific for HIV-I antigens.

BACKGROUND OF THE INVENTION


Lentivirus-like particles are extremely helpful as antigen presenting and delivery system as well as to detect antibodies in human sera, including antibodies directed against conformational epitopes. VLPs can be constructed and purified by a variety of methods, but most often a viral expression system such as baculovirus or vaccinia that effectively produce lentiviral particles is used. Virus-like particles (VLPs) represent an antigen presenting and delivery system currently under investigation as potential vaccine for different human viruses, such as Hepatitis, Papilloma, Rotavirus, Parvovirus and Norwalk virus (Gamier, L. et al., Journal of Virology 69: 4060-4068, 1995; Guerrero, R. A. et al., Journal of Virology 75: 9713-9722, 2001; Kirnbauer, R. et al., Proceedings of National Academy of Science USA 89: 12180-12184, 1992; Li, T. C et al., Journal of Virology 71: 7207-13., 1997; O'Neal, C. M. et al., Journal of Virology 71: 8707-8717, 1997; Sedlik, C. et al., Proc.Natl.Acad.Sci.U.S.A. 94: 7503-7508, 1997). In particular, HIV-targeted VLPs (HIV-VLPs) are based on the HIV-I Pr55gag precursor protein property to assemble as immature, non-replicating and non-infectious VLPs with an effective induction of both arms of the immune response (Buonaguro, L. et al., Antiviral Research 49: 35-47, 2001; Buonaguro, L. et al., Antiviral

The HIV-VLP_A show a strong in vivo immunogenicity in Balb/c mice, in absence of adjuvants, and HIV-I-specific CTLs as well as cross-clade neutralizing antibodies, active on primary HIV-I isolates, have been detected in immunized animals (Buonaguro, L. et al., Antiviral Research 54: 189-201, 2002). Furthermore, the immunogenicity of the HIV-VLP_A has been evaluated in Balb/c mice by intra-nasal (i.n.) routes, in a homologous (VLP+VLP) prime-boost protocol without any adjuvant formulation, showing the induction of humoral IgA and/or IgG antibody response with neutralizing activity, in sera and at mucosal (vaginal and intestinal) sites, as well as cellular immune response (Buonaguro, L. et al., Journal of Virology 79: 7059-7067, 2005).

The HIV-VLP_A immunogenicity has been further evaluated in a Balb/c mouse model by intra-nasal administration, comparing a VLP+VLP homologous vs a DNA+VLP heterologous prime-boost immunization protocol and evaluating the enhancing effect of the Eurocine L3 mucosal adjuvant. The results showed that the non-adjuvanted heterologous protocol induced a statistically significant increase of serum anti-env Ig titers, compared to the homologous protocol, and that the single adjuvanted HIV-VLP_A boost, either in the homologous or in the heterologous protocol, induced a relevant increase of the serum anti-gag response. This effect was less dramatic for the anti-env response but a statistically significant increase of serum IgA was observed in the heterologous protocol. Similar results were observed at mucosal (vaginal and intestinal) sites (Buonaguro, L. et al., Vaccine 25: 5968-5977, 2007).

The envelope glycoprotein (Env) complex of human immunodeficiency virus type 1 (HIV-I) is made of two components: the surface gpl20 and the trans-membrane gp41, synthesized as a gpl60 precursor that is cleaved within the cell (McCune, J. M. et al., Cell 53: 55-67, 1988; Moulard, M. et al., Biochim.Biophys.Acta 1469: 121-132, 2000). This is generally considered to be a trimeric structure, containing three gpl20 and three gp41 moieties held together by noncovalent interactions. The native Env complex is unstable, because the noncovalent intersubunit interactions that hold gpl20 to gp41 are weak, as are the intermolecular
interactions between the gp41 moieties (Eckert, D. M. et al, Annu.Rev.Biochem. 70: 777-810, 2001; Poignard, P. et al., Annu.Rev.Immunol. 19: 253-274, 2001; Wyatt, R. et al., Science 280: 1884-1888, 1998). This instability is probably essential for receptor-triggered conformational changes to occur, but it does cause a problem for attempts to express the native complex as a recombinant protein (Binley, J. M. et al., Journal of Virology 74: 627-643, 2000). The lability of the noncovalent interaction between gp120 and the gp41 ectodomain can be overcome by introducing a correctly positioned intermolecular disulfide bond to make a soluble form of Env, SOS gpl40. In the presence of cotransfected furin, the peptide bond linking gp120 to gp41ECTO is cleaved, allowing the production of properly processed gpl40 (Binley, J. M. et al., Journal of Virology 74: 627-643, 2000; Sanders, R. W. et al., Journal of Virology 74: 5091-5100, 2000). To further stabilize the gp41-gp41 interactions in SOS gpl40, an amino acid substitution has been introduced into the N-terminal heptad repeat region. One such soluble protein, SOS I559P gpl40 (designated SOSIP gpl40), is properly folded, proteolytically cleaved, substantially trimeric, and has appropriate receptor binding and antigenic properties (Sanders, R. W. et al., Journal of Virology 76: 8875-8889, 2002).

Recently, VLPs have been produced in mammalian cell lines expressing on the surface env-trimers made of SOS gp120-gp41 (Crooks, E. T. et al., Virology 366: 245-262, 2007). However, the efficiency of surface expression is significantly related to the compatibility between the cellular expression system and the amino acid composition of the signal sequence (SS) and the trans-membrane (TM) sequences. In fact, the expression levels of the HIV-I envelope protein in different heterologous systems appear to be significantly low (Barr, P. J. et al., J.BioLChem. 263: 16471-8., 1988; Chakrabarti, S. et al., Nature. 320: 535-7., 1986; Crowl, R. et al., Gene. 38: 31-8., 1985; Dewar, R. L. et al., Journal of Virology 63: 2452-6., 1989; Lasky, L. A. et al., Science. 233: 209-12., 1986). This may represent a successful feature of the HIV-I which, presenting a limited amount of the coat glycoprotein on the infected cells, hampers the neutralizing response mounted by the immune system. Within the HIV-I env glycoprotein signal sequence, the unusual highly charged region (Lasky, L. A. et al., Science. 233: 209-12., 1986; Ratner, L. et al., Nature. 313: 277-84., 1985) has been demonstrated to be responsible for the poor expression and secretion of gp120, prolonging the retention in the endoplasmic reticulum (ER) (Li, Y. et al., Virology. 204: 266-78., 1994; Li, Y. et al., Proc.Natl.Acad.Sci.U.S.A. 93: 9606-11., 1996). In particular, the substitution of the autologous signal sequence (ASS) with the Honeybee Mellitin signal sequence (HMSS) has
resulted in a dramatic increase of HIV-1 gpl20 expression, glycosylation and secretion from the SF9 insect cells (Li, Y. et al, Virology, 204: 266-78., 1994). Moreover, the TM domain of the baculovirus major glycoprotein gp64 is critical for protein trafficking to the plasma membrane, membrane fusion, and virion budding in SF9 insect cells. These features are extremely relevant to significantly improve the budding of the VLPs from the plasma membrane (Li, Z. et al., Journal of Virology 82: 3329-3341, 2008).

Mucosal surfaces are surface areas that are vulnerable to infection by pathogenic microorganisms. The adaptive immune system is designed to distinguish antigens, pathogens and vaccines that enter the body through mucosal surfaces from those that are introduced directly into tissues or the bloodstream by injection or injury. In this framework, it is becoming increasingly clear that local mucosal immune responses are important for protection against disease (i.e. mucosal antibodies against Vibrio cholerae bacteria). Mucosal immune responses are most efficiently induced by the administration of vaccines onto mucosal surfaces, whereas injected vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infection at mucosal surfaces (Lamm, M. E.Annu.Rev.Microbiol. 51: 311-340, 1997; Levine, M. M.J.Pediatr.Gastroenterol.Nutr. 31: 336-355, 2000). Nevertheless, clinical vaccine research has been based largely on injection of antigens, and most vaccines in use today are administered intramuscularly or subcutaneously and only a few mucosal vaccines have been approved for human use worldwide. These include oral vaccines against polio virus (Modlin, J. F.JAMA 292: 1749-1751, 2004), Salmonella typhi (Levine, M. M.J.Pediatr.Gastroenterol.Nutr. 31: 336-355, 2000), V. cholerae (Levine, M. M.J.Pediatr.Gastroenterol.Nutr. 31: 336-355, 2000) and rotavirus (Kapikian, A. Z. et al., J.Infect.Dis. 174 Suppl 1: S65-S72, 1996) and a nasal vaccine against influenza virus (Belshe, R. B. et al., N.Engl.J.Med. 338: 1405-1412, 1998).

Mucosal surfaces are separated from the outside by epithelial barriers which produce non-specific or innate defences including mucins and antimicrobial proteins. Epithelial cells are active participants in mucosal defence. They function as sensors that detect dangerous microbial components through patternrecognition receptors such as Toll-like receptors (TLRs). They respond by sending cytokine and chemokine signals to underlying mucosal cells, such as dendritic cells (DCs) and macrophages, to trigger innate, non-specific defences and promote adaptive immune responses (Izadpanah, A. et al., Am.J.Physiol GastrointestXiver Physiol 280: G710-G719, 2001; Kagnoff, M. F. et al., J.Clin.Invest 100: 6-10, 1997). Therefore, mucosal tissues are in a constant state of alert, but they are adapted to the presence of foreign microorganisms and their products. As a result, vaccines that would
produce vigorous immune responses if injected into a sterile environment, such as muscle, might be 'ignored' when given mucosally, where the tissue is constantly exposed to microorganisms.

An important characteristic of the mucosal adaptive immune response is the local production and secretion of dimeric or multimeric immunoglobulin A (IgA) antibodies that, unlike other antibody isotypes, are resistant to degradation in the protease-rich external environments of mucosal surfaces.

The protease resistance of secretory IgA (slgA) is a result of its dimerization and high degree of glycosylation during its synthesis in mucosal plasma cells, and its association with a glycosylated fragment (the secretory component) derived from the epithelial polymeric immunoglobulin receptor (plgR) that mediates transport of dimeric IgA across epithelial cells to the lumen (Kaetzel, C. S. et al., Proc.Natl.Acad.Sci.U.S.A 88: 8796-8800, 1991). slgA has multiple roles in mucosal defence, promoting the entrapment of antigens or microorganisms in the mucus, preventing direct contact of pathogens with the mucosal surface, a mechanism that is known as 'immune exclusion'. Alternatively, slgA of the appropriate specificity might block or sterically hinder the microbial surface molecules that mediate epithelial attachment (Hutchings, A. B. et al., Journal of Virology 78: 947-957, 2004), or it might intercept incoming pathogens within epithelial-cell vesicular compartments during plgR-mediated transport (Kaetzel, C. S. et al., Proc.Natl.Acad.Sci.U.S.A 88: 8796-8800, 1991; Lamm, M. E.Annu.Rev.Microbiol. 51: 311-340, 1997). In particular, VLPs are most likely phagocytosed by microfold epithelial cells (M cells) in the nasal lumen and then directly deposited to the NALT (nasal associated lymphoid tissue) via M cell transcytosis (Wu, H. Y. et al., Immunol.Res. 16: 187-201, 1997), which preferentially drains into lymph nodes. This process induces strong local (NALT) and distant immune responses in both peripheral and mucosal immune compartments. Soluble antigens bypass the NALT and are directly fed into superficial lymph nodes by antigen presenting cells in the nasal lumen resulting in a lower local immune response.

Within the different mucosal administration routes, the sublingual immunotherapy (SLIT) have examined its safety as well efficacy, although the underlying immunological mechanisms are not yet fully understood (Moingeon, P. et al., Allergy 61: 151-165, 2006). Sublingual administration leads to slower absorption into the bloodstream, and leads to the presentation of dendritic cells in the oral mucosa and/or in the regional lymph nodes of the neck. Compared with skin dendritic cells, those in the oral mucosa have increased expression of major histocompatibility complex class I and II molecules and of costimulatory molecules,
such as CD40, B7.1, and B7.2. These findings imply that dendritic cells in the oral mucosa are especially adept at antigen presentation, and may play an important role in directing subsequent immune effects with induction of specific systemic as well as mucosal IgG and IgA (Huang, C. F. et al, Ann.Allergy Asthma Immunol. 99: 443-452, 2007). This administration route has been prevalently used as an effective therapeutic tool for the treatment of respiratory allergic disorders (Bousquet, J.Arb.Paul Ehrlich Inst.Bundesamt Sera Impfstoffe Frankf A M.237-241, 2006).

Considering that the transmission of HIV-I infection during heterosexual or homosexual intercourse accounts for as much as 80% of AIDS globally, a specific mucosal immunity is extremely relevant for controlling the primary HIV-I transmission. This can be achieved by mucosally delivered vaccines which, besides the advantage of conferring mucosal as well as systemic immunity, show an increased stability and allow the elimination of needles. This, especially in developing countries, may significantly reduce the risk of further spreading HIV-I infection. Moreover, the immunogenic efficacy of mucosal vaccines would greatly benefit from the co-administration with mucosal adjuvants that can initiate and support the transition from innate to adaptive immunity.

**SUMMARY OF THE INVENTION**

The present invention is directed to a method of expressing an HIV-I gag-based Virus-Like Particles structure presenting on the surface a modified HIV-I envelope protein in a cell, comprising transfecting the cell with an expression vector containing the HIV-I capsid protein coding sequence under conditions facilitating expression of the protein in the cell.

In an aspect of the invention, there is provided the description of a modified gpl40 envelope protein containing adapted signal sequence (SS) and trans-membrane (TM) regions for an optimized intra-cellular transportation toward the VLP surface.

In a preferred embodiment of the invention, there is provided a method of expressing the gag capsid protein coding sequence of HIV-I in Sf-9 insect cells using the baculovirus expression system. The HIV-I coding sequences were cloned using standard techniques in the art into a baculovirus transfer vector. The same baculovirus transfer vector contained also coding sequences of the modified HIV-I envelope gpl40 molecule from a subtype A, Ugandan isolate. The resulting baculovirus transfer vector were used to transform modified E coli bacteria cells, in order to obtain a recombinant baculovirus bacmid DNA to transfect Sf-9 insect cells for the expression of the protein in the cells. It was shown that the gag protein formed virus-like particles (VLPs) presenting on their surface the modified SOSIP gpl40 (SOSIPgpl40- VLPs). VLPs were identified by electron microscopy of negatively-stained
sucrose band fractions obtained from Sf-9 cells infected with the recombinant baculovirus. It was further discovered that the VLPs possessed immunological and morphological characteristics similar to those of native HIV-I virions, as defined by mice antisera.

Virus-like particle(s) produced in accordance with the invention, can be used in diagnostic assays and can be used for vaccine development (both therapeutic and prophylactic). It is understood that the method of the invention as described herein for production of HIV-VLPs can be used to produce similar immunologic reagents from other HIV-I subtypes and/or CRFs. In addition, VLPs produced in accordance with the invention will provide abundant reagents with which to carry out immunologic studies of HIV-I and for developing vaccines against HIV-I.

The present invention also provides a method of vaccinating a mammal against HIV-I infection by administering HIV-I virus-like particles mucosally (including sublingual) to a mammal in an amount sufficient to induce an immune response to the HIV-I antigens.

**BRIEF DESCRIPTION OF THE FIGURES**

**FIG. 1A** shows the modified HIV-I gpl40 envelope gene containing the Honeybee Mellitin Signal Sequence (HBMSS) and the Trans-Membrane domain of gp64.

**FIG. 1B** shows the schematic representation of the pFastBac Dual™ vector, expressing both the HIV-I gag and the modified HBM-SOSIPgpl40-gp64TM envelope genes.

**FIG. 2A** shows the density of HBM-SOSIPgpl40-gp64TM-VLPs produced by infected Sf9 insect cells. Supernatants from cultures of HighFive insect cells, infected with recombinant baculovirus (M.O.I.-5), were harvested 96 h post-infection. VLPs were sedimented through a continuous sucrose gradient (10-60%) and 20 aliquots of 500 μl were collected. The gag content was measured by a commercial p24 capture assay and antigenic peaks were observed in the fractions 14-17 spanning the 1.14-1.18 g:ml density interval.

**FIG. 2B** shows a Western blot analysis of the p24 reactive fractions. Proteins were separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane and reacted with a 1:250 dilution of human sera from HIV-I seropositive subject. Arrows indicate the specific reactive HIV-I viral proteins; molecular weight marker positions are shown on the left and expressed in kilodaltons (kD).

**FIG. 3** shows the trimerization of the modified HBM-SOSIPgpl40-gp64TM derived from VLPs in a Blue-Native PAGE and subsequent Western blot analysis, probing with mouse polyclonal antiserum specific for HIV-I specific epitopes. Bis-Tris gels in native (blue-native) (Lane 1) and denaturing reducing conditions (Lane 2). Ferritin was used as a molecular weight marker with bands at 439 and 220 kDa, as indicated.
FIG. 4 shows an electron micrograph of HBM-SOSIPgpl40-gp64TM-VLPs budding from Sf9 insect cells.

FIGS. 5 collectively show the immune response at systemic and mucosal level induced in mice by HBM-SOSIPgpl40-gp64TM-VLPs (20µg) by intra-nasal (i.n.) and sub-lingual (s.l.) administration. Fig. 5A (serum); Fig 5B (fecal); Fig. 5C (vaginal). Pre- and post-immune sera were evaluated in an ELISA specific for HBM-SOSIPgpl40-gp64TM-VLPs.

Fig. 6 shows the T helper cell activation in mice immunized with HBM-SOSIPgpl40-gp64TM-VLPs (20µg) by intra-nasal (i.n.) and sub-lingual (s.l.) administration. The panel shows the Stimulation Index (S.I.) pattern of splenocytes from the PBS control and the immunized groups, stimulated with increasing doses of rgpl20MN (Intracell). S.I. represents the fold increase of thymidine incorporation in cells from immunized animals compared to cells from control animals. The mean value obtained for each animal group in two independent experiments is shown; the standard error of the means was always lower than 10% of the mean value.

Fig. 7 Cellular immunity: IFNγ production. Spleen cells from immunized mice were isolated and cultured for 3 days in the presence of env peptides. Results are represented as the IFNγ produced by individual animals and mean titers±S.E.M. are indicated for each animal group.

Fig. 8 Ex vivo heterologous neutralization analysis. The HIV-I neutralization assay was performed on PHA-stimulated PBMCs using two heterologous A (92UG031) and B viral (SF2) isolates. 30 TCID50 of each isolate were pretreated with serial dilutions of the immune sera, collected at the end of the complete immunization schedule. The individual neutralization activity of serum antibodies, at 1:20, 1:40 and 1:80 dilutions, is represented as percentage of the virus replication compared to control samples. Mean titers±S.E.M. are indicated for each animal group. A 50% neutralization (dotted line) has been considered the lowest limit to score positive the test.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention relates generally to HIV-I. More particularly, the invention relates to a method of 1) expressing the human HIV-I major capsid protein coding sequence using several expression systems, including the eukaryotic baculovirus and the vegetal expression systems; 2) designing a modified HIV-I gpl40 envelope linked to heterologous signal sequence and trans-membrane regions; 3) production of HIV-I virus-like particles expressing on the surface a modified HIV-I gpl40 (HBM-SOSIPgpl40-gp64TM-VLPs) and use of these VLPs for mucosal (including sublingual) immunization of mammals to induce systemic and mucosal (Vaginal and rectal) immune response specific for HIV-I antigens.
As used herein, "virus-like particle(s) (VLPs)" refer to a virus-like particle(s), fragment(s), capsomer(s) or portion(s) thereof produced from the capsid protein coding sequence of HIV-I and comprising antigenic characteristic(s) similar to those of infectious HIV-I particles. As used herein, "antigenic characteristic(s)" refers to

1. the ability of the virus-like particle(s) to cross-react with wild-type particles (native infectious virus particles of the same HIV type) as determined by antisera generated in animals and/or humans by immunization with either VLPs or infectious virus; and/or

2. the ability to recognize or detect antibodies in human sera from persons known to be infected with homologous virus.

As used herein, "gag protein coding sequence" or "gag capsid protein coding sequence" or "gag coding sequence" refers to the open reading frame which codes for the gag protein in HIV-I. When expressed, the gag protein coding sequence produces a protein, or protein complex, or aggregate, which possesses immunological and morphological characteristics similar to those of native HIV-I virions. The gag coding sequence used in the invention can be isolated and purified from HIV-I genomic DNA or synthesized using standard genetic engineering techniques.

As used herein, the term "transfecting" refers to any means for introducing a virus, plasmid or vector into a cell. Examples of such means include infection, calcium phosphate precipitation and electroporation.

In a preferred embodiment of the invention, there is provided a method of expressing the coding sequence for the gag capsid protein of human immunodeficiency 1 , together with the coding sequence of modified HIV-I gpl40 envelope linked to heterologous signal sequence and trans-membrane regions, in Sf-9 insect cells using the baculovirus expression system. It is understood that the capsid protein coding sequences of these HIV-I types are used for purposes of illustration only, and that any gag and envelope protein coding sequences for any HIV-I subtype and CRFs can be used without deviating from the intended scope of the invention. Such HIV-I subtypes and CRFs include, without limitation, all those already discovered and described (Buonaguro, L. et al, Journal of Virology 81: 10209-10219, 2007) and all those to be discovered and described in the future.

The preferred expression system used in the method of the invention is the baculovirus expression system, however, it is understood that any other expression system(s) can be employed herein provided the system(s) can express the gag protein coding sequence. Examples of such systems include, without limitation, any prokaryotic and/or eukaryotic
system(s) including adenovirus, SV40, E. coli, CHO cells, vaccinia virus, insect viruses, yeast, bacteriophage virus or modified viruses, DNA plasmids, vectors and the like.

The host cell for expression of the gag coding sequence is dependent on the expression system used. Examples of suitable host cells include, without limitation, bacteria (prokaryotic), microorganisms such as yeast, mammalian cells (eukaryotic) and insect cells. When using the baculovirus expression system insect cells, such as Sf-9 or Sf-21 are preferred.

In another aspect of the invention, it has been designed a modified HIV-I gpl40 envelope protein made of the HIV-I gpl20 and the ectodomain of the gp41 linked at the NH$_2$ with a signal sequence of the Honeybee Mellitin (HBMSS) and at the COOH with the transmembrane region (TM) of the baculovirus gp64 (HBM-SOSIPgpl40-gp64TM). The ectodomain of the gp41 included in the modified gpl40 is responsible for the formation of trimers and the amino acid substitutions in the gpl40 sequence (SOSIPgpl40) stabilize the trimeric structure (Binley, J. M. et al. Journal of Virology 74: 627-643, 2000; Sanders, R. W. et al., Journal of Virology 74: 5091-5100, 2000). The HBMSS efficiently increase of HIV-I gpl20 expression, glycosylation and secretion from the SF9 insect cells and TM domain of the baculovirus major glycoprotein gp64 improves protein trafficking to the plasma membrane, membrane fusion, and virion budding in SF9 insect cells.

In one embodiment of the invention, there is provided a method of expressing the gag major capsid protein of HIV-I and the HBM-SOSIPgpl40-gp64TM in several expression systems, including Sf-9 insect cells using the baculovirus expression system, to obtain HIV virus-like particles (HBM-SOSIPgpl40-gp64TM- VLPs). It was further discovered that the HBM-SOSIPgpl40-gp64TM-VLPs show antigenic characteristics similar to those of native infectious HIV-I particles and are recognized by sera from HIV-I seropositive individuals.

In another aspect of the invention, it was discovered that the HBM-SOSIPgpl40-gp64TM-VLPs comprise antigenic characteristic(s) similar to those of native infectious HIV-I particles. For example, reaction of VLP-containing insect cell extracts with antisera directed against either denatured or non-denatured capsid epitopes, as deduced by immunoreactivities in Western blot assays, suggested that conformational epitopes present in native HIV-I infectious virions were also present on the baculovirus-produced HBM-SOSIPgpl40-gp64TM-VLPs of the present invention. The HBM-SOSIPgpl40-gp64TM envelope protein expressed on the VLP surface shows the ability to be conformationally similar (including multimer formation), as on the native infectious HIV-I particles.
These morphologic and immunologic similarities to native HIV virions suggest that recombinant VLPs produced in the baculovirus system will be useful in sero-epidemiology and pathogenesis studies of HIV-I infections as well as for vaccine development. The VLPs of the invention can be used to raise antibodies, either in subjects for which protection against infection by HIV-I is desired, i.e., preventive vaccines, or to heighten the immune response to an HIV-I infection already present, i.e. therapeutic vaccines.

The VLPs of the invention can be directly administered to a host to induce the formation of neutralizing antibodies, to confer either protective immunity against HIV-I or, if the patient is already infected, to boost the patient's own immune response. For all applications, the HBM-SOSIPgp140-gp64TM-VLPs are administered in immunogenic form. Optionally, the HBM-SOSIPgp140-gp64TM-VLPs can be conjugated to an immunogenicity conferring carrier material, the material preferably being antigenically neutral. Depending on the use required, the VLPs of the invention have the ability to serve as type specific or broad range vaccines and diagnostics.

VLPs which are to be administered as vaccines can be formulated according to conventional and/or future methods for such administration to the subject to be protected and can be mixed with conventional adjuvants. The peptide expressed can be used as an immunogen in subunit vaccine formulations, which may be multivalent. The multivalent vaccine formulation can comprise VLPs each encoding a different gag and envelope protein from different HIV-Is. The product may be purified for purposes of vaccine formulation from any vector/host systems that express the heterologous protein. The purified VLPs should be adjusted to an appropriate concentration, formulated with any suitable vaccine adjuvant and packaged for use. Suitable adjuvants include, but are not limited to: mineral gels, e.g., aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols; polyanions; peptides; oil emulsions; and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum. The immunogen may also be incorporated into liposomes, or conjugated to polysaccharides and/or other polymers for use in a vaccine formulation. Many mucosal methods may be used to administer the vaccine formulations described above, these include, but are not limited to, oral, sublingual, intranasal, intravaginal routes. If they are to be used directly, as diagnostic reagents, they are purified using conventional methods and packaged accordingly for such use. If they are to be used to produce antibodies for diagnostic purposes, convenient test animals can be used to prepare the appropriate antisera. Suitable hosts include mice, rats, rabbits, guinea pigs, or even larger mammals such as sheep. The antibodies can be used therapeutically so long as they are
compatible with the host to be treated. Monoclonal antibodies having the proper species characteristics are preferred for this application.

In a preferred embodiment, the invention provides a method of vaccinating a mammal for HIV-I by administering HBM-SOSIPgp140-gp64TM-VLPs mucosally to a mammal in an amount sufficient to induce an immune response to the HIV-I. In order to obtain a high degree of protection, the method may also involve administering one or more vaccine booster inoculations of HBM-SOSIPgp140-gp64TM-VLPs mucosally to the mammal.

The present invention also provides oral vaccines having HIV-I virus-like particles and a pharmaceutically acceptable carrier. Oral vaccines may also include flavorings, colorings, and other food additives to make the vaccine more palatable. In addition, oral vaccines may also contain stabilizers and preservatives to extend the shelf life of the vaccine. The present results demonstrate that systemic and mucosal (vaginal and rectal) responses can be induced by mucosal (intra-nasal and sublingual, in particular) HBM-SOSIPgp140-gp64TM-VLP immunization. Antigenic specificities of orally induced antibodies were found to be dependent on native VLP structure. Results from ex vivo neutralization assays indicated that post-immune serum and mucosal antibodies efficiently inhibited cross-clade HIV-I infection of target cells. This demonstrates the usefulness of HBM-SOSIPgp140-gp64TM-VLPs as oral immunogens for the prevention of systemic as well as mucosal HIV-I transmission.

Mucosal, including sublingual, immunization offers certain advantages over other routes of vaccination. For example, mucosal vaccines are more easily administered and thus may be more acceptable to vaccine recipients. Also, mucosal vaccines can be less pure than vaccines formulated for injection, making production costs lower. Moreover, mucosally administered antigens have been shown to elicit mucosal immune responses, which may be important for protection against infection with certain pathogens.

In particular, sublingual routes of administration have certain advantages over simple oral (swallowing) or gastrointestinal (GI) administration. This route is often faster, and entering a macromolecule or a drug into one's body sublingually ensures that the substance will rapidly come in contact with the sublingual epithelium prior to entry into the bloodstream. The responses induced by sublingual routes of administration were comparable to those seen after nasal immunization with similar doses of antigen and adjuvant. Importantly, and akin to nasal immunization, sublingual immunization induced antigen-specific cytolytic T cell responses in the lungs and in the genital tract. Moreover, sublingual immunization induced vigorous systemic humoral and CTL responses at doses comparable to those required for nasal
administration. Such systemic responses include cell-mediated immune responses that elicit production of interferon gamma by T-lymphocyte cells. However, development of nasal vaccination strategies in humans may be possibly hampered by undesirable side effects, including those on the central nervous system through interaction of various antigens including live viruses and recombinant adjuvants with the olfactory epithelium (van Ginkel, F. W. et al, Journal of Immunology 165: 4778-4782, 2000; van Ginkel, F. W. et al, Infect. Immun. 73: 6892-6902, 2005).

Over 25 million individuals are estimated to have died of AIDS since the start of the pandemic. Up to 38.6 million individuals are currently infected with HIV, the majority living in sub-Saharan Africa and Asia. An estimated 4.1 million individuals were newly infected with HIV in 2005, 95% of whom are living in developing countries (UNAIDS. 2006 Report on the global AIDS epidemic. Geneva: UNAIDS; 2006). Considering that the transmission of HIV-I infection during heterosexual or homosexual intercourse accounts for as much as 80% of AIDS globally, a specific mucosal immunity is extremely relevant for controlling the primary HIV-I transmission. This can be achieved by mucosally delivered vaccines which, besides the advantage of conferring mucosal as well as systemic immunity, show an increased stability and allow the elimination of needles. This, especially in developing countries, may significantly reduce the risk of further spreading HIV-I infection. Therefore, efficient methods of vaccine delivery will be needed for the immunization of large numbers of susceptible individuals. Thus, mucosal, including sublingual immunization strategies will certainly facilitate implementation of mass immunization programs designed to reduce the incidence of HIV-I infections.

The following Examples are provided to further illustrate the present invention.

**EXAMPLE 1**

**Methods**

1. **Design and synthesis of the HBM-SOSIPgpl40-gp64TM molecule.**

   The SOSIPgpl40, based on the envelope sequence from the 94UG018 Ugandan HIV-I A-clade isolate (Buonaguro, L. et al., AIDS Research and Human Retroviruses 14: 1287-1289, 1998) has been designed as previously described (Binley, J. M. et al., Journal of Virology 74: 627-643, 2000; Sanders, R. W. et al., Journal of Virology 76: 8875-8889, 2002) introducing the G511C, I569P and P615C substitutions. These modifications have been shown to stabilize both the intra-molecular gpl20-gp41 link and the formation of trimers by soluble gpl40s (Binley, J. M. et al., Journal of Virology 74: 627-643, 2000; Sanders, R. W. et al., Journal of Virology 76: 8875-8889, 2002).
Innovatively, the SOSIPgpl40 have been added two regions relevant for efficiently driving the molecule toward the cell surface (Honeybee Mellitin Signal Sequence, HBMSS at NH\textsubscript{2} terminus) and displaying on the cell membrane (gp64 trans-membrane, gp64TM, at the COOH terminus). The gp64TM, moreover, is relevant also for an efficient budding of virus-like particles from the Sf9 insect cells.

2. **Cloning of the HBM-SOSIPgpl40-gp64TM into pFast Bac Dual Baculovirus Transfer Vector.**

The HBM-SOSIPgpl40-gp64TM was digested with MMI at 5' and Na\textsubscript{10} at 3' ends to be cloned into the corresponding sites in pFast Bac Dual Baculovirus Transfer Vector, downstream of the pLo promoter, and positive clones were screened for the correct orientation relative to the driving promoter. The pFast Bac Dual Baculovirus Transfer Vector already contains a HIV-I gag-pol-nef sequence subcloned downstream of the pPolh promoter (Buonaguro, L. et al., Antiviral Research 49: 35-47, 2001).

3. **Generation of recombinant Baculovirus DNA.**

Recombinant bacmids were obtained, in the DHlOBac\textsuperscript{Tm} E. coli (Invitrogen), by site-specific transposition of the mini-Tn7 element from the recombinant pFastBac Dual vector to the mini-attTn7 attachment site on the bacmid, with the Tn7 transposition functions provided in trans by the pMON7124 helper plasmid. The transposition of viral genes on the bacmid disrupts the lacZa reading frame, resulting in white bacterial colonies over a background of blue colonies that harbor the parental bacmid. The recombinant bacmid DNA was isolated from bacteria following a protocol specifically developed for large plasmids (>100 kb) (Ioannou et al., 1994). The insertion of viral genes was verified by the polymerase chain reactions (PCR) performed with pUC:M13 universal primers, located on either side of the mini-attTn7 sequence of the bacmid, paired with internal primers specific for the transposed sequences: (5'-GGCCCATAGGAAAAAGGGCTTGTGG-3') for the Pr55gag sequence, and (5'-TGTTAAACCGACGGCATTGCTTGAATGCGAGTCGCTACG-3') for the gpl20 sequence. All PCR amplifications were performed in 1.5 mM MgCl\textsubscript{2} and were based on 30 cycles with an elongation step of 5 min at 72°C.

4. **SDS-PAGE and Western Blot detection of recombinant gag and env expression in Sf-9 Cells.**

*Spodoptera frugiperda* Sf-9 insect cells were propagated in TC100 medium supplemented with 10% FCS (Gibco-BRL) and 9x10\textsuperscript{5} cells seeded in a 6-well plate were transfected with 10-20 µg of recombinant bacmid DNA, by the Cellfectin method.
(Gibco-BRL). For large-scale preparations of VLPs, HighFive cells, derived from *Trichoplusia* ni egg cell homogenates (Invitrogen Inc.) and propagated in serum-free SF900 medium (Gibco-BRL), were infected with the recombinant baculovirus, released in the Sf9 cell culture supernatants, at a multiplicity of infection (m.o.i.) of 5. Both insect cell lines were propagated at 28°C in the absence OfCO₂. Cells and supernatants from Sf9 or HighFive cell cultures were collected 4 days post-transfection: infection. Lysates were prepared by boiling cellular pellets for 5 min in a buffer containing 5% b-mercaptoethanol, 2% SDS, 10% glycerol and 0.01% bromophenol blue.

The denatured samples were loaded in a single 100 mm wide sample well, separated by electrophoration in a 10% SDS polyacrylamide gel, and analyzed by conventional Western Blot technique (Sambrook, J. et al., 2: 1989). Immobilized proteins were incubated with a 1:200 dilution of mouse anti-gpl20:V3 or anti-p24 MAb (Intracel Co.) and, subsequently, with biotin-conjugated anti-mouse IgG antibodies (1:1500 dilution). Alternatively, the proteins were first incubated with a 1:1000 dilution of human sera from HIV-I seropositive individuals and, subsequently, with biotin-conjugated anti-human IgG antibodies (1:1500 dilution) The bound molecules were visualized by the addition of horseradish peroxidase (HRPO)-conjugated streptavidine and the chromogen 4-Chloro-1-Naphtol.

Production and Purification of HIV Virus-Like Particles.

Recombinant VLPs were purified directly from the cell-free culture supernatant. In particular, supernatants were clarified by centrifugation at 2000x g for 15 min at 4°C and VLPs were pelleted by ultra-centrifugation at 100000 x g for 75 min through a 25% sucrose cushion, as previously described (Rovinski et al., 1992). VLPs pelleted from cell culture supernatants by ultra-centrifugation were suspended in TNE solution, layered onto a continuous sucrose gradient (10-60%) and centrifuged at 100000xg for 1.5 h at 4°C in a Beckman SW41 rotor. Gradient fractions were collected from the bottom of the tubes in 500 μl aliquots and the VLPs quantified by a commercial p24-antigen capture assay (NENDupont). Gardient fractions containing the highest amount of p24 antigen were separated by SDS-PAGE and analyzed by conventional Western Blot technique. Immobilized proteins were incubated with a 1:200 dilution of mouse anti-gpl20:V3 or anti-p24 MAb (Intracel Co.) and, subsequently, with biotin-conjugated anti-mouse IgG antibodies (1:1500 dilution). The bound molecules were visualized by the addition of horseradish peroxidase (HRPO)-conjugated streptavidine and the chromogen 4-Chloro-1-Naphtol.
6. Characterization of gpl40 trimers on the surface of VLPs.
   To analyze Env derived from VLPs under native conditions, a BN-PAGE protocol has
   been performed as previously described (Moore, P. L. et al, Journal of Virology 80:
   2515-2528, 2006). In particular, VLPs were incubated in an equal volume of
   solubilization buffer (0.12% Triton X-100 in 1 mM EDTA/1.5 M aminocaproic acid)
   and one microliter of a protease inhibitor cocktail (Sigma). An equal volume of 2x
   sample buffer containing 100 mM morpholinepropanesulfonic acid (MOPS), 100 mM
   Tris-HCl, pH 7.7, 40% glycerol, and 0.1% Coomassie blue was then added. Samples
   were loaded onto a 4 to 12% Bis-Tris NuPAGE gel (Invitrogen). Samples were
   electrophoresed at 4°C for 3 h at 100 V with 50 mM MOPS/50 mM Tris, pH 7.7,
   containing 0.002% Coomassie blue as cathode buffer and the same buffer without
   Coomassie blue as the anode buffer. The gel was then Western blotted onto
   polyvinylidene difluoride. Excess Coomassie blue dye was removed after blotting by
   washing with 30% methanol/10% acetic acid then 100% methanol. The blot was then
   transferred to blocking buffer (4% nonfat milk in PBS) for 30 min and probed using 1
   µg/ml of anti-gpl20 2G12 MAb, or 20 µg/ml of anti-gp41 4E10 MAb. Goat anti-human
   and/or mouse Fc and Fab’2 alkaline phosphatase conjugates were used, as appropriate,
   to detect the primary MAbs at 1:3,000 (Jackson).

EXAMPLE II

Electron microscopic visualization of VLPs

Electron micrographs of negatively stained preparations of sucrose banded HBM-
SOSIPgpl40-gp64TM-VLPs (FIGS. 4) showed distinct VLPs. Particle diameters determined
by direct measurement of the VLPs in FIG. 4, were approximately 100 nm.

EXAMPLE III

Orally administered VLPs induce systemic immunoglobulin G (IgG) and IgA antibody
responses.

1. Immunization experiments in mice and collection of immune samples.
   Female BALB/c mice, each group consisting of three animals 6 to 8 weeks of age, were
   administered 20 µg purified VLPs by the i.n., or sub-lingual route. Control mice were
   treated with endotoxin-free phosphate-buffered saline (PBS). The immunization
   schedule was based on a 4-dose regimen, where the booster inoculations were
   administered at weeks 3, 7, and 9 after the primary vaccine dose. All the immunization
   protocols were performed in two independent experiments without addition of
   adjuvants.
(a) i.n. vaccination. Prior to inoculation, mice were anesthetized by halothane. Animals were treated by placing 200 μl of inoculum, 50 μl at time, into alternating nostrils and allowing mice to inhale. Animals were monitored until consciousness was regained.

(b) Sub-lingual vaccination. Mice were anesthetized with halothane and 5 μl of the solution was administered with a pipette under the tongue. The mice were then maintained 30 minutes without food and water. No adverse signs were noted in any mice receiving i.n. or s.l. inoculation.

2. Sample collection and processing.

(a) Serum samples. Blood samples were collected from each animal 1 week after each vaccination dose by puncture of the retro-orbital vein. After the last dose, the entire blood volume was collected by tail bleeding; serum was obtained by standard methods and stored at -80°C until use.

(b) Vaginal secretions. Vaginal secretions were collected from each animal 1 week after each vaccination dose by pipetting 50 μl of PBS in and out of the vagina gently until a discrete clump of mucus was recovered. This usually took four to eight cycles of pipetting and required cutting the pipette tip back to a diameter of 1 to 2 mm. A second vaginal wash with 50 μl of PBS was then done to ensure more complete recovery of the vaginal secretions, and this material was combined with the first wash. Vaginal washes were centrifuged at 12,000 x g for 10 min shortly after collection to separate the mucus from the PBS wash solution. The mucus and supernatant were then frozen separately at -80°C. The PBS wash solution contained a cocktail of proteinase inhibitors (153.8 nM aprotinin, 3.2 μM bestatin, and 10 μM leupeptin). In order to obtain complete recovery of slgA and presumably also IgG from the vaginal mucus, samples were thawed, weighed, and extracted twice for 2 h each time in 100 μl of PBS per sample, with rotation at 20 rpm in a 12-ml polystyrene tube at 4°C. The two extracts and the original wash supernatant were pooled, made up to 300 μl per sample, and frozen at -80°C until needed (48).

(c) Fecal pellets. Fecal antibody samples were collected from each mouse 1 week after each vaccination dose. Fecal pellets were resuspended at a 10% (wt/vol) concentration in a stool diluent (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM CaCl2, 0.05% Tween 20, 5 mM sodium azide, supplemented with 1 mg of aprotinin/ml and 10 mg of leupeptin/ml). After incubation on ice for 20 min, suspensions were
centrifuged at 18,000 x g for 15 min in order to remove fecal solids. Processed fecal antibody samples were stored at -80°C.

3. **Serological assays.**

The presence and the titers of IgG and IgA antibodies, specific for the native conformational HBM-SOSIPgpl40-gp64TM-VLPs used in the immunization protocols, were evaluated in samples obtained from immunized mice by an enzyme-linked immunosorbent assay (ELISA). Ninety-six-well MICROTEST assay plates (Beckton Dickinson) were coated with 2.5 µg of HBM-SOSIPgpl40-gp64TM-VLPs. Antigens were allowed to adhere to the plates by incubation at +4°C overnight, and nonspecific protein binding to the plates was blocked by incubation with 5% (wt/vol) dry milk in PBS for 1 h at room temperature (RT). Diluted mouse sera or mucosal fluids were added to the wells and incubated for 1 h at RT. After three washes, wells were treated for 1 h at RT with peroxidise-conjugated goat anti-mouse IgG or IgA; after three additional washes, the reaction was visualized with 0.075% 4-chloro-l-naphthol in 0.056% hydrogen peroxide and stopped with 2 N sulfuric acid. Absorbance was determined at 492 nm, and reactions were considered positive when the mean absorbance for immunized animals exceeded the mean absorbance of equal dilutions of sera collected from control animals by a factor of 3. Serum ELISA results are expressed as the log10 geometric mean titer of the last positive dilution point from the animals of each set in the two independent experiments. Results for antibodies in vaginal or fecal samples are expressed as the optical density (OD) obtained with 1:5-diluted fluids.

**EXAMPLE IV**

Cell mediated immunity.

Lymphocyte proliferation and cytokine production. For lymphocyte proliferation assay, single-cell suspensions were prepared from spleen and 4x10^5 cells/per well were co-cultured with antigen (0.1 µg rgpl20, ProteinSciences, CT per well) for 3 days before tritium-H3-labeled thymidine (1 µCi/ml) was added to each well and incubated for 16 h. The cells were harvested and counted in a β-counter (WallacI450, Finland). After 72 h, interferon-γ was determined in the culture supernatants by ELISA in accordance with the manufacturer’s instructions (R&DSystems, UK).

**EXAMPLE V**

HIV-1 neutralization assay.

Serum from preimmunized and immunized mice were heat-inactivated (56°C for 30 min) and serially diluted at 3-fold dilutions, starting at 1/20. Virus aliquots of the dualtropic T cell line-
adapted HIV-I SF2 strain and the primary NSI/CCR5 tropic clade A isolate 92UG031 were diluted in medium supplemented with 10% inactivated FCS (Invitrogen Life Technologies, Paisley, U.K.). 10 IU/ml IL-2 (Amersham Biosciences, Little Chalfont, U.K.), 50µg/ml streptomycin, and 50 IU penicillin (Invitrogen Life Technologies). Seventyfive microliters of each virus dilution, corresponding to 30 MOI for the SF2 strain and to 30 MOI for the 92UG031 strain, and 75 µl of each serum dilution were incubated in duplicate for 1 h at 37°C in round-bottom culture plates (Nunc). Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation and seventy-five microliters containing 1210⁶/ml PHA-stimulated PBMC were added to each well. After 16- to 18-h incubation at 37°C, the cells were washed with RPMI 1640, and 200 µl of fresh medium was added to each well. Every 3 days, 100 µl of medium was changed. After 6-7 days, 100µl of supernatant from each well was collected, and virus production was measured in a p24 Ag capture ELISA.

The present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, however, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.
CLAIMS

I. A method of inducing an immune response in a mammal against HIV-I comprising:
administering HIV-I virus-like particles mucosally, including sublingual, to a
mammal in an amount sufficient to induce an immune response to the HIV-I.

2. The method according to claim 1 further comprising:
administering one or more vaccine booster inoculations of HIV-I virus-like particles
mucosally, including sublingual, to the mammal.

3. The method according to claim 1, wherein the HIV-I is a field isolate HIV-I.

4. The method according to claim 1, wherein the HIV-I virus-like particles express the
wild-type or modified HIV-I major capsid protein.

5. The method according to claim 1, wherein the HIV-I virus-like particles express the
wild-type or modified HIV-I gp140 envelope protein or fragments of it (peptides).

6. The method according to claim 5, wherein the modified HIV-I gp140 envelope
protein is made of the HIV-I gpl20 and the ectodomain of the gp41 linked at the NH$_2$
with a signal sequence of the Honeybee Mellitin (HBMSS) and at the COOH with the
trans-membrane region (TM) of the baculovirus gp64 (HBM-SOSIPgpl40-gp64TM).

7. The method according to claim 3, wherein the HIV-I virus-like particles are
administered as a pharmaceutically acceptable formulation.

8. The method according to claim 3, wherein the HIV-I virus-like particles are
administered in an acceptable adjuvanted formulation.

9. The method according to claim 3, wherein the HIV-I virus-like particles are
administered alone or in a heterologous prime-boost immunization strategy.

10. The method according to claim 3, wherein the mucosally administered HIV-I virus-
like particles induce systemic and mucosal (Vaginal and rectal) immune response
specific for HIV-I antigens.

II. VLPs presenting trimeric and conformational molecules, including HIV-I envelopes,
wherein the HIV-I virus-like particles express the wild-type or modified HIV-I gp140
envelope protein or fragments of it (peptides).

12. VLPs according to claim 11, wherein the modified HIV-I gp140 envelope protein is
made of the HIV-I gpl20 and the ectodomain of the gp41 linked at the NH$_2$ with a
signal sequence of the Honeybee Mellitin (HBMSS) and at the COOH with the trans-
membrane region (TM) of the baculovirus gp64 (HBM-SOSIPgpl40-gp64TM).
FIG. 3

FIG. 4
FIG. 5A
FIG. 5B
FIG. 5C
FIG. 6

T-helper

Stimulation Index (S.i.)

rgp120 (ug)

i.n.  s.l.  PBS

FIG. 7

IFN gamma

pg/ml

i.n.  s.l.  PBS
FIG. 8
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K39/21

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

**B. DOCUMENTS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K

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

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

EPO-Internal, BIOSIS, WPI Data, MEDLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<tr>
<th>Category</th>
<th>Citation of document with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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<tr>
<td>X</td>
<td>YUKI YOSHIKAZU ET AL: &quot;Progress towards an AIDS mucosal vaccine: An overview&quot; TUBERCULOSIS (AMSTERDAM), vol. 87, no. Suppl. 1, August 2007 (2007-08), pages S35-S44, XP002515144 ISSN: 1472-9792 the whole document ----- -/--</td>
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Further documents are listed in the continuation of Box C

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- T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- A' document member of the same patent family

Date of the actual completion of the international search

16 February 2009

Date of mailing of the international search report

27/02/2009

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**DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>A</td>
<td>LI YAN ET AL: &quot;Control of Expression, Glycosylation, and Secretion of HIV-I gp120 by Homologous and heterologous Signal Sequences&quot; VIROLOGY, vol. 204, no. 1, 1994, pages 266-278, XP002467778 ISSN: 0042-6822 cited in the application the whole document</td>
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<td>A</td>
<td>WANG BAO-ZHONG ET AL: &quot;Incorporation of high levels of chimeric human immunodeficiency virus envelope glycoproteins into virus-like particles&quot; JOURNAL OF VIROLOGY, vol. 81, no. 20, October 2007 (2007-10), pages 10869-10878, XP002515146 ISSN: 0022-538X the whole document</td>
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