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(54) Title: MUCOSAL BOOSTING FOLLOWING PARENTERAL PRIMING

(57) Abstract: Mucosal immunization using one or more antigens following parenteral administration of the same or different antigens is described.

MUCOSAL BOOSTING FOLLOWING PARENTERAL PRIMING

Technical Field

The present invention relates generally to mucosal immunization of one or more antigens following parenteral administration of the same or different antigens. Use of these mucosal boosting systems for inducing immune responses following is also described.

Background of the Invention

Development of vaccines that invoke immunity, particularly mucosal immunity, against various pathogens would be desirable. Many disease-causing pathogens, such as bacteria, viruses, parasites and other microbes, are transmitted through mucosal surfaces.

One example of a virus thought to be transmitted through mucosal surfaces is acquired immune deficiency syndrome (AIDS). AIDS is recognized as one of the greatest health threats facing modern medicine and worldwide sexual transmission of HIV is the leading cause of AIDS. There are, as yet, no cures or vaccines for AIDS.

In 1983-1984, three groups independently identified the suspected etiological agent of AIDS. See, e.g., Barre-Sinoussi et al. (1983) Science 220:868-871; Montagnier et al., in Human T-Cell Leukemia Viruses (Gallo, Essex & Gross, eds., 1984); Vilmer et al. (1984) The Lancet 1:753; Popovic et al. (1984) Science 224:497-500; Levy et al. (1984) Science 225:840-842. These isolates were variously called lymphadenopathy-associated virus (LAV), human T-cell lymphotropic virus type III (HTLV-III), or AIDS-associated retrovirus (ARV). All of these isolates are strains of the same virus, and were later collectively named Human Immunodeficiency Virus (HIV). With the isolation of a related AIDS-causing virus, the strains originally called HIV are now termed HIV-1 and the related virus is called HIV-2 See, e.g., Guyader et al. (1987) Nature 326:662-669; Brun-Vezinet et al. (1986) Science 233:343-346; Clavel et al. (1986) Nature 324:691-695. Consequently, there is a need in the art for compositions and methods suitable for treating and/or preventing HIV infection worldwide.

A great deal of information has been gathered about the HIV virus, and several targets for vaccine development have been examined including the *env*, *Gag*, *pol* and *tat* gene products encoded by HIV. Immunization with native and synthetic HIV-encoding polynucleotides has also been described, as described for example, in co-owned PCT/US99/31245 and references cited therein. In addition, polynucleotides encoding HIV have been administered in various attempts to identify a vaccine. (*See, e.g.,* Bagarazzi et al. (1999) *J. Infect. Dis.* 180:1351-1355; Wang et al. (1997) *Vaccine* 15:821-825). A replication-competent Venezuelan equine encephalitis (VEE) alphavirus vector carrying the matrix/capsid domain of HIV could elicit CTL responses has been administered subcutaneously in animals (Caley et al. (1997) *J. Virol.* 71:3031-3038). In addition, alphavirus vectors derived from Sindbis virus has also been shown to elicit HIV gag-specific responses in animals (Gardner et al. (2000) *J. Virol.* 74:11849-11857). Similarly, HIV peptides have also been administered to animal subjects. (Staats et al. (1997) *AIDS Res Hum Retroviruses* 13:945-952; Belyakov (1998) *J.Clin.Invest.* 102: 2072).

One example of a bacteria that may be transmitted through mucosal surfaces is *Neisseria meningitidis* (*N. meningitidis* or N.men.). *Neisseria meningitidis* a causative agent of bacterial meningitis and sepsis. Meningococci are divided into serological groups based on the immunological characteristics of capsular and cell wall antigens. Currently recognized serogroups include A, B, C, W-135, X, Y, Z and 29E. The polysaccharides responsible for the serogroup specificity have been purified from several of these groups, including A, B, C, W-135 and Y. See, also, WO 00/66791; WO 99/24578; WO 00/71574; WO 99/36544; WO 01/04316; WO 99/57280; WO 01/31019; WO 00/22430; WO 00/66741; WO 00/71725; WO 01/37863; WO 01/38350; WO 01/52885; WO 01/64922; WO 01/64920; WO 96/29412; and WO 00/50075.

N. meningitidis serogroup B (termed "MenB" or "NmB" herein) accounts for a large percentage of bacterial meningitis in infants and children residing in the U.S. and Europe. The organism also causes fatal sepsis in young adults. In adolescents, experimental MenB vaccines consisting of outer membrane protein (OMP) vesicles are somewhat protective. However, no protection has been observed in vaccinated infants, the age group at greatest risk of disease. Additionally, OMP vaccines are serotype- and

subtype-specific, and the dominant MenB strains are subject to both geographic and temporal variation, limiting the usefulness of such vaccines.

Effective capsular polysaccharide-based vaccines have been developed against meningococcal disease caused by serogroups A, C, Y and W135. In addition, a combination MenB/MenC vaccine has been described. See, WO 99/61053. However, similar attempts to develop a MenB polysaccharide vaccine have failed due to the poor immunogenicity of the capsular MenB polysaccharide (termed "MenB PS" herein). MenB PS is a homopolymer of (N-acetyl (α 2–>8) neuraminic acid. *Escherichia coli* K1 has the identical capsular polysaccharide. Antibodies elicited by MenB PS cross-react with host polysialic acid (PSA). PSA is abundantly expressed in fetal and newborn tissue, especially on neural cell adhesion molecules ("NCAMs") found in brain tissue. PSA is also found to a lesser extent in adult tissues including in kidney, heart and the olfactory nerve. Thus, most anti-MenB PS antibodies are also autoantibodies. Such antibodies therefore have the potential to adversely affect fetal development, or to lead to autoimmune disease.

MenB PS derivatives have been prepared in an attempt to circumvent the poor immunogenicity of MenB PS. For example, C_3-C_8 N-acyl-substituted MenB PS derivatives have been described. See, EP Publication No. 504,202 B, to Jennings et al. Similarly, U.S. Patent No. 4,727,136 to Jennings et al. describes an N-propionylated MenB PS molecule, termed "NPr-MenB PS" herein. Mice immunized with NPr-MenB PS glycoconjugates were reported to elicit high titers of IgG antibodies. Jennings et al. (1986) *J. Immunol.* 137:1708. In rabbits, two distinct populations of antibodies, purportedly associated with two different epitopes, one shared by native MenB PS and one unshared, were produced using the derivative. Bactericidal activity was found in the antibody population that did not cross react with MenB PS. Jennings et al. (1987) *J. Exp. Med.* 165:1207. The identity of the bacterial surface epitope(s) reacting with the protective antibodies elicited by this conjugate remains unknown. Also, because a subset of antibodies elicited by this vaccine has autoreactivity with host polysialic acid (Granoff et al. (1998) *J. Immunol.* 160:5028) the safety of this vaccine in humans remains uncertain. Thus, it is readily apparent that the production of a safe and effective vaccine against MenB would be particularly desirable.

Cancer (tumor) antigens form yet another broad class of antigens for which it would be desirable to have safe and effective vaccines. (See, *e.g.,* Moingeon (2000) *Vaccine* 19:1305-1326; Rosenberg (2001) *Nature* 411:380-384). Various tumor-specific antigens have been identified and attempts have been made to develop vaccines based on whole cells or uncharacterized tumor lysates. Moingeon, *supra*. However, there are currently no proven vaccines for various cancers.

Certain prime-boost methods of immunization have been described. In particular, genetic immunizations involving polynucleotides as have been described. (See, *e.g.,* WO 01/81609; WO 00/11140; Cooney et al. (1993) *Proc Nat'l Acad Sci U S A* 90(5):1882-1886, describing induction of an immune response by intramuscular priming with a recombinant vaccinia (vac/env) virus expressing HIV-1 envelope and intramuscular boosting with a gp160 glycoprotein derived from a recombinant baculovirus (rgp160); Bruhl et al. (1998) *AIDS Res Hum* Retroviruses 14:401-407, describing mucosal priming with recombinant vaccinia followed by parenteral priming; and Eo et al. (2001) *J. Immunol.* 166:5473-5479, describing mucosal prime and mucosal boost with recombinant vaccinia virus expressing the gB protein of HSV). Lee et al. (1999) *Vaccine* 17:3072-3082, describes mucosal prime and parenteral boosting regimes using recombinant *Helicobacter pylori* urease vaccine.

However, despite these and other studies, there remains a need for compositions and methods of enhancing mucosal and systemic immunity to various antigens, including to pathogens or cancers for which there are currently few or no effective vaccines and/or treatments.

Summary of the Invention

The present invention provides methods for generating an immune response in a mammal by parenteral priming followed by mucosal boosting.

In one aspect, a method of generating an immune response in a subject is described. The method comprises (a) parenterally administering a first immunogenic composition comprising one or more polypeptide antigens and; (b) mucosally administering a second immunogenic composition comprising one or more antigens, thereby inducing an immune response in the subject.

In another aspect, a method of generating an immune response against a tumor antigen is described, the method comprising the steps of (a) parenterally administering a first immunogenic composition comprising one or more tumor antigens and; (b) mucosally administering a second immunogenic composition comprising one or more tumor antigens.

The mucosal administration can be, for example, intrarectal, intravaginal or intranasal. Further, in any of the methods described herein, parenteral administration can be, for example, transcutaneous. The first and/or second immunogenic compositions can further comprise one or more additional agents such as adjuvants and/or delivery vehicles, for example microparticles such as PLG.

In certain embodiments, at least one antigen is derived from a bacteria, for example, *Neisseria meningitidis,* subgroups A, B and or C (*e.g.,* capsular oligosaccharide antigens alone or conjugated to CRM197); *Haemophilus influenzae, Streptococcus pneumoniae*, *Streptococcus agalactiae.* In other embodiments, at least one antigen is derived from a virus, for example, hepatitis A virus (HAV), human immunodeficiency virus (HIV), respiratory syncytial virus (RSV), parainfluenza virus (PIV), influenza, hepatitis B virus (HBV), herpes simplex virus (HSV), hepatitis C virus (HCV) and/or human papilloma virus (HPV). In yet other embodiments, at least one antigen is derived from a tumor.

In any of the methods described herein, the immune response can be humoral and/or cellular and, furthermore, can be a systemic immune response (*e.g.,* IgG production), a mucosal immune response (*e.g.,* IgA production) or a combination of systemic and mucosal responses. The methods described herein can be used to generate an immune response to one or more pathogens (*e.g.,* bacteria, viruses, tumors, etc.).

In any of the methods described herein the first and second immunogenic compositions can comprise antigens from the same pathogen (*e.g.,* bacteria, virus and/or tumor). In certain embodiments, the first and second immunogenic compositions are the same. In other embodiments, the first and second immunogenic compositions are different, for example by having different antigens from the same pathogen, different forms of the antigens, antigens from different pathogens and/or different adjuvants.

In any of the methods described herein, the immunogenic compositions comprise, entirely or partially, one or more polynucleotides encoding one or more

antigens. In certain embodiments, the first immunogenic composition further comprises at least one polynucleotide encoding one or more antigens. In other embodiments, all or some of the antigens of the second immunogenic are encoded by one or more polynucleotides.

Further, in any of the methods described herein, the methods described herein further comprise repeating step (a) and/or step(b) one or more times. In certain aspects, step (b) is performed two or more times. The time interval between the mucosal administrations of step (b) can be hours, days, months or years. Further, in certain embodiments, the repeated steps are performed using the same or, alternatively, different, immunogenic compositions.

Thus, it is an object of the invention to provide alternative and improved methods for mucosal boosting following parenteral priming of an immune response. The invention provides a method for raising an immune response in a mammal, the method comprising the parenteral administration of a first immunogenic composition followed by the mucosal administration of a second immunogenic composition. The mucosal administration further comprises the use of a mucosal adjuvant, for example, CpG containing oligos, bioadhesive polymers, or *E. coli* heat-labile entertoxin ("LT") or detoxified mutants thereof or cholera toxin ("CT") or detoxified mutant thereof or microparticles that are formed from materials that are biodegradeable and non-toxic. The parenteral administration preferably further comprises the use of a parenteral adjuvant, for example alum, and the like. In certain embodiments, microparticles are used for the delivery of the immunogenic composition(s).

The first immunogenic composition is given parenterally. Suitable routes of parenteral administration include intramuscular, subcutaneous, intravenous, intraperitoneal, intradermal, transcutaneous, or transdermal routes as well as delivery to the interstitial space of a tissue. In one embodiment, parenteral priming is via the intramuscular route. The first immunogenic composition is preferably adapted for parenteral administration in the form of an injectable that will typically be sterile and pyrogen-free. (See, e.g., WO 99/43350). In certain embodiments, the first immunogenic composition comprises a parenteral or immunological adjuvant. In addition, the first immunogenic composition may be adsorbed onto microparticles that are biodegradeable and non-toxic. The second immunogenic composition is given

mucosally. Suitable routes of mucosal administration include oral, intranasal, intragastric, pulmonary, intestinal, rectal, ocular and vaginal routes. Intranasal or oral administration is preferred.

In certain aspects, the second immunogenic composition is preferably adaptable for mucosal administration. Where the composition is for oral administration, it may be in the form of tablets or capsules, optionally enteric-coated, liquid, transgenic plants etc. Where the composition is for intranasal administration, it may be in the form of a nasal spray, nasal drops, gel or powder. In certain embodiments, the second immunogenic composition further comprises a mucosal adjuvant. Suitable adjuvants include: CpG containing oligo, bioadhesive polymers, see WO 99/62546 and WO 00/50078; *E. coli* heat-labile entertoxin ("LT") or detoxified mutants thereof or cholera toxin ("CT") or detoxified mutant thereof or microparticles that are formed from materials that are biodegradeable and non-toxic. Preferred LT mutants include K63 or R72. See e.g., PCT EP92/03016; PCT IB94/00068; PCT IB96/00703 and PCT IB97/00183.

In other aspects the first and/or second immunogenic compositions are adsorbed to microparticles. In certain embodiments, the microparticles used in the first and/or second immunogenic composition are 100 nm to 150 nm in diameter, more preferably 200 nm to 30 μm in diameter and most preferably 500 nm to 10 μm in diameter and are made from for example, poly(alpha-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride a polycaprolactone etc. See e.g., WO 00/06123 and WO 98/33487.

Immunogenic compositions suitable for use in the present invention include proteins of, and/or polynucleotides encoding, viral, bacterial, parasitic, fungal and/or cancer antigens.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.).

Brief Description of the Drawings

Figure 1 is a graph depicting enhancement of serum and vaginal antibody responses against HIV envelope peptides following systemic prime and mucosal boost immunizations. The diagonal stripes bars show serum antibody while the gray bars

show titers from vaginal washes. The various modes of delivery and adjuvants are indicated on below the bars on the horizontal axis.

Figure 2 is a graph depicting HIV envelope-specific serum IgG titers (as measured by ELISA) with a single intramuscular (IM) or intranasal (IN) memory boost 18 months after original prime-boost. The various modes of delivery and adjuvants are indicated below the bars on the horizontal axis.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); *Methods In Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Handbook of Surface and Colloidal Chemistry* (Birdi, K.S. ed., CRC Press, 1997); *Short Protocols in Molecular Biology*, 4th ed. (Ausubel et al. eds., 1999, John Wiley & Sons); *Molecular Biology Techniques: An Intensive Laboratory Course*, (Ream et al., eds., 1998, Academic Press); *PCR (Introduction to Biotechniques Series)*, 2nd ed. (Newton & Graham eds., 1997, Springer Verlag); Peters and Dalrymple, *Fields Virology* (2d ed), Fields et al. (eds.), B.N. Raven Press, New York, NY.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, for example, reference to “an antigen” includes a mixture of two or more such agents.

Prior to setting forth the invention definitions of certain terms that will be used hereinafter are set forth.

A "polynucleotide" is a nucleic acid molecule that encodes a biologically active (e.g., immunogenic or therapeutic) protein or polypeptide. Depending on the nature of the polypeptide encoded by the polynucleotide, a polynucleotide can include as little as

10 nucleotides, e.g., where the polynucleotide encodes an antigen. Furthermore, a "polynucleotide" can include both double- and single-stranded sequences and refers to, but is not limited to, cDNA from viral, prokaryotic or eukaryotic MRNA, genomic RNA and DNA sequences from viral (e.g. RNA and DNA viruses and retroviruses) or prokaryotic DNA, and especially synthetic DNA sequences. The term also captures sequences that include any of the known base analogs of DNA and RNA, and includes modifications such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the nucleic acid molecule encodes a therapeutic or antigenic protein. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the antigens. Modifications of polynucleotides may have any number of effects including, for example, facilitating expression of the polypeptide product in a host cell.

The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the proteins or errors due to PCR amplification. Furthermore, modifications may be made that have one or more of the following effects: reducing toxicity; facilitating cell processing (*e.g.*, secretion, antigen presentation, etc.); and facilitating presentation to B-cells and/or T-cells.

A "fusion molecule" is a molecule in which two or more subunit molecules are linked, preferably covalently. The subunit molecules can be the same chemical type of molecule, or can be different chemical types of molecules. Examples of the fusion molecules include, but are not limited to, fusion polypeptides (for example, a fusion between two or more antigens) and fusion nucleic acids (for example, a nucleic acid encoding the

fusion polypeptides described herein). See, also, Sambrook et al., *supra* and Ausubel et al., *supra* for methods of making fusion molecules.

An "antigen" refers to a molecule containing one or more epitopes (either linear, conformational or both) that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is used interchangeably with the term "immunogen." Normally, an epitope will include between about 3-15, generally about 5-15 amino acids. A B-cell epitope is normally about 5 amino acids but can be as small as 3-4 amino acids. A T-cell epitope, such as a CTL epitope, will include at least about 7-9 amino acids, and a helper T-cell epitope at least about 12-20 amino acids. Normally, an epitope will include between about 7 and 15 amino acids, such as, 9, 10, 12 or 15 amino acids. The term "antigen" denotes both subunit antigens, (i.e., antigens which are separate and discrete from a whole organism with which the antigen is associated in nature), as well as, killed, attenuated or inactivated bacteria, viruses, fungi, parasites or other microbes as well as tumor antigens, including extracellular domains of cell surface receptors and intracellular portions that may contain T-cell epitopes. Antibodies such as anti-idiotype antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the definition of antigen as used herein. Similarly, an oligonucleotide or polynucleotide that expresses an antigen or antigenic determinant *in vivo*, such as in gene therapy and DNA immunization applications, is also included in the definition of antigen herein.

Epitopes of a given protein can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Nat'l Acad Sci. USA* 81:3998-4002; Geysen et al. (1986) *Molec. Immunol* 23:709-715.

Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols*, supra.

For purposes of the present invention, antigens can be derived from tumors and/or any of several known viruses, bacteria, parasites and fungi, as described more fully below. The term also intends any of the various tumor antigens or any other antigen to which an immune response is desired. Furthermore, for purposes of the present invention, an "antigen" refers to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the ability to elicit an immunological response, as defined herein. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the antigens.

An "immunological response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to an antigen present in the composition of interest. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, including secretory (IgA) or IgG molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells ("CTL"s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells. In addition, a chemokine response may be induced by various white blood or endothelial cells in response to an administered antigen.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376. Recent methods of measuring cell-mediated immune response include measurement of intracellular cytokines or cytokine secretion by T-cell populations (*e.g.,* by ELISPOT technique), or by measurement of epitope specific T-cells (e.g., by the tetramer technique)(reviewed by McMichael, A.J., and O'Callaghan, C.A., *J. Exp. Med.* 187(9):1367-1371, 1998; Mcheyzer-Williams, M.G., et al, *Immunol. Rev.* 150:5-21, 1996; Lalvani, A., et al, *J. Exp. Med.* 186:859-865, 1997).

Thus, an immunological response as used herein may be one that stimulates CTLs, and/or the production or activation of helper T- cells. The production of chemokines and/or cytokines may also be stimulated. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an immunological response may include one or more of the following effects: the production of antibodies (*e.g.,* IgA or IgG) by B-cells ; and/or the activation of suppressor, cytotoxic, or helper T-cells and/or γδ T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art.

An "immunogenic composition" is a composition that comprises an antigenic molecule where administration of the composition to a subject results in the development in the subject of a humoral and/or a cellular immune response to the antigenic molecule of interest. The immunogenic composition can be introduced

directly into a recipient subject, such as by injection, inhalation, oral, intranasal or any other parenteral or mucosal (*e.g.*, intra-rectally or intra-vaginally) route of administration.

By "subunit vaccine" is meant a vaccine composition that includes one or more selected antigens but not all antigens, derived from or homologous to, an antigen from a pathogen of interest such as from a virus, bacterium, parasite or fungus. Such a composition is substantially free of intact pathogen cells or pathogenic particles, or the lysate of such cells or particles. Thus, a "subunit vaccine" can be prepared from at least partially purified (preferably substantially purified) immunogenic polypeptides from the pathogen, or analogs thereof. The method of obtaining an antigen included in the subunit vaccine can thus include standard purification techniques, recombinant production, or synthetic production.

By "parenteral" is meant introduction into the body outside the digestive tract, such as by subcutaneous, intramuscular, transcutaneous, intradermal or intravenous administration. This is to be contrasted with delivery to a mucosal surface, such as oral, intranasal, vaginal or rectal. Thus, "mucosal" is meant introduction into the body via any mucosal surface, such as intranasally, orally, vaginally, rectally or the like.

By "co-administration" is meant introduction into a body or target cell of two or more compositions. The term includes administration in any order or concurrently.

The term "microparticle" as used herein, refers to a particle of about 100 nm to about 150 μm in diameter, more preferably about 200 nm to about 30 μm in diameter, and most preferably about 500 nm to about 10 μm in diameter. Preferably, the microparticle will be of a diameter that permits parenteral administration without occluding needles and capillaries. Microparticle size is readily determined by techniques well known in the art, such as photon correlation spectroscopy, laser diffractometry and/or scanning electron microscopy.

Microparticles for use herein will be formed from materials that are sterilizable, non-toxic and biodegradable. Such materials include, without limitation, poly(α-hydroxy acid), polyhydroxybutyric acid, polycaprolactone, polyorthoester, polyanhydride. Preferably, microparticles for use with the present invention are derived from a poly(α-hydroxy acid), in particular, from a poly(lactide) ("PLA") or a copolymer of D,L-lactide and glycolide or glycolic acid, such as a poly(D,L-lactide-co-glycolide)

("PLG" or "PLGA"), or a copolymer of D,L-lactide and caprolactone. The microparticles may be derived from any of various polymeric starting materials that have a variety of molecular weights and, in the case of the copolymers such as PLG, a variety of lactide:glycolide ratios, the selection of which will be largely a matter of choice, depending in part on the co administered antigen. These parameters are discussed more fully below.

An "immuno-modulatory factor" refers to a molecule, for example a protein that is capable of modulating (particularly enhancing) an immune response. Non-limiting examples of immunomodulatory factors include lymphokines (also known as cytokines), such as IL-6, TGF-β, IL-1, IL-2, IL-3, etc.); and chemokines (*e.g.,* secreted proteins such as macrophage inhibiting factor). Certain cytokines, for example TRANCE, flt-3L, and a secreted form of CD40L are capable of enhancing the immunostimulatory capacity of APCs. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1α), interleukin-11 (IL-11), MIP-1γ, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO), CD40 ligand (CD40L), tumor necrosis factor-related activation-induced cytokine (TRANCE) and flt3 ligand (flt-3L). Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA). The sequences of many of these molecules are also available, for example, from the GenBank database. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or mutants thereof) and nucleic acid encoding these molecules are intended to be used within the spirit and scope of the invention. Immunomodulatory factors can be included with one, some or all of the compositions described herein or can be employed as separate formulations.

By "subject" is meant any member of the subphylum chordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals

including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The system described above is intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

By "vertebrate subject" is meant any member of the subphylum cordata, including, without limitation, mammals such as cattle, sheep, pigs, goats, horses, and humans; domestic animals such as dogs and cats; and birds, including domestic, wild and game birds such as cocks and hens including chickens, turkeys and other gallinaceous birds. The term does not denote a particular age. Thus, both adult and newborn animals are intended to be covered.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, i.e., the material may be administered to an individual in a formulation or composition without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

The terms "effective amount" or "pharmaceutically effective amount" of a macromolecule and/or microparticle, as provided herein, refer to a nontoxic but sufficient amount of the macromolecule and/or microparticle to provide the desired response, such as an immunological response, and corresponding therapeutic effect, or in the case of delivery of a therapeutic protein, an amount sufficient to effect treatment of the subject, as defined below. As will be pointed out below, the exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the condition being treated, and the particular macromolecule of interest, mode of administration, and the like. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the microparticle formulation without causing

any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

By "physiological pH" or a "pH in the physiological range" is meant a pH in the range of approximately 7.2 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

As used herein, "treatment" refers to any of (i) the prevention of infection or reinfection, as in a traditional vaccine, (ii) the reduction or elimination of symptoms, and (iii) the substantial or complete elimination of the pathogen or disorder in question. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

A. ANTIGENS

The parenteral prime-mucosal boost methods described herein can involve parenteral and mucosal administration of one or more antigens (or polynucleotides encoding these antigens). For purposes of the present invention, virtually any polypeptide or polynucleotide can be used. Antigens can be derived from any of several known viruses, bacteria, parasites and fungi, as well as any of the various tumor antigens or any other antigen to which an immune response is desired. Furthermore, for purposes of the present invention, an "antigen" refers to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the ability to elicit an immunological response. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the antigens. Antigens that are particularly useful in the practice of the present invention include polypeptide antigens derived from pathogens that infect or are transmitted through mucosal surfaces. Non-limiting representative examples of pathogens transmitted through mucosal surfaces and antigens derived therefrom include antigens derived from bacterial pathogens (*e.g., Neisseria meningitidis, Streptococcus agalactia, Haemophilus influenzae, Streptococcus pneumoniae,* chlamydia, gonorrhea and syphilis), viral pathogens (*e.g.*, Human Immunodeficiency Virus ("HIV"), Hepatitis B and C Virus ("HBV" and "HCV", respectively), Human Papiloma Virus ("HPV"), Herpes Simplex Virus ("HSV"), and the like), as well as parasitic, fungal and cancer

antigens. For a discussion of *Chlamydia pneumoniae* and *Chlamydia trachomatis,* see Kalman et al. (1999) *Nature Genetics* 21:385-389; Read et al. (2000) *Nucleic Acids Research* 28:1397-1406; Shirai et al. (2000) *J. Infect. Dis.* 181(Suppl.3):S524-S527; WO 99/27105; WO 00/27994; WO 00/37494; WO 99/28457.

As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen that is capable, under the appropriate conditions, of causing an immune response (*i.e.*, cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatability Complex ("MHC") class I presentation, MHC Class II presentation, or both. As will be evident to one of ordinary skill in the art, various immunogenic portions of the antigens described herein may be combined in order to induce an immune response when administered as described herein.

Furthermore, the immunogenic portion(s) may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing computer algorithms such as TSITES (MedImmune, Maryland), in order to scan coding regions for potential T-helper sites and CTL sites. From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA, which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays and proliferation assays.

Immunogenic portions of any antigen may also be selected by other methods. For example, the HLA A2.1 transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T cell receptor repertoire recognizes the same antigenic determinants recognized by human T cells. In both systems, the CTL response generated in the HLA A2.1 transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al. (1991) *J. Exp. Med. 173*:1007-1015; Vitiello et al. (1992) *Abstract of Molecular Biology of Hepatitis B Virus Symposia*).

Additional immunogenic portions may be obtained by truncating the coding sequence at various locations including, for example, to include one or more epitopes from the various regions, for example, of the HIV genome or one or more MenB epitopes. As noted above, such domains include structural domains such as *Gag*, *Gag-polymerase, Gag-protease, reverse transcriptase (RT), integrase (IN)* and *Env.* The structural domains are often further subdivided into polypeptides, for example, p55, p24, p6 (*Gag*); p160, p10, p15, p31, p65 (*pol, prot, RT and IN)*; and gp160, gp120 and gp41 (*Env*). Additional epitopes of HIV and other sexually transmitted diseases are known or can be readily determined using methods known in the art. Also included in the invention are molecular variants of such polypeptides, for example as described in PCT/US99/31245; PCT/US99/31273 and PCT/US99/31272.

Antigens may be used alone or in any combination. (See, *e.g.,* WO 02/00249 describing the use of combinations of bacterial antigens). The combinations may include multiple antigens from the same pathogen, multiple antigens from different pathogens or multiple antigens from the same and from different pathogens. Thus, bacterial, viral, tumor and/or other antigens may be included in the same composition or may be administered to the same subject separately. It is generally preferred that combinations of antigens be used to raise an immune response be used in combinations. Immunization against multiple pathogens or antigens is advantageous, both for parenteral delivery (where the number of administrations is reduced) but it is less important in mucosal vaccines (*e.g.* intranasal vaccines) and for mucosal delivery because patient compliance is improved and transport/storage of medicines is facilitated. Furthermore, the immunization(s) as described herein can be used either prophylatically or therapeutically.

1. Antigens derived from Bacteria

The invention described herein will also find use with numerous bacterial antigens, such as those derived from organisms that cause diphtheria (*See, e.g.,* Chapter 3 of *Vaccines*, 1998, eds. Plotkin & Mortimer (ISBN 0-7216-1946-0), staphylococcus (*e.g., Staphylococcus aureus* as described in Kuroda et al. (2001) *Lancet* 357:1225-1240), cholera, tuberculosis, *C. tetani,* also known as tetanus (*See, e.g.,* Chapter 4 of *Vaccines*, 1998, eds. Plotkin & Mortimer (ISBN 0-7216-1946-0), Group A and Group B

streptococcus (including *Streptococcus pneumoniae, Streptococcus agalactiae* and *Streptococcus pyogenes* as described, for example, in Watson et al. (2000) *Pediatr. Infect. Dis. J.* 19:331-332; Rubin et al. (2000) *Pediatr Clin. North Am.* 47:269-284; Jedrzejas et al. (2001) *Microbiol Mol Biol Rev* 65:187-207; Schuchat (1999) *Lancet* 353:51-56; GB patent applications 0026333.5; 0028727.6; 015640.7; Dale et al. (1999) *Infect Dis Clin North Am* 13:227-1243; Ferretti et al. (2001) *PNAS USA* 98:4658-4663), pertussis (*See, e.g.,* Gusttafsson et al. (1996) *N. Engl. J. Med.* 334:349-355; Rappuoli et al. (1991) *TIBTECH* 9:232-238), meningitis, *Moraxella catarrhalis* (*See, e.g.,* McMichael (2000) *Vaccine* 19 Suppl. 1:S101-107) and other pathogenic states, including, without limitation, Neisseria meningitides (A, B, C, Y), *Neisseria gonorrhoeae* (*See, e.g.,* WO 99/24578; WO 99/36544; and WO 99/57280), Helicobacter pylori (*e.g.,* CagA, VacA, NAP, HopX, HopY and/or urease as described, for example, WO 93/18150; WO 99/53310; WO 98/04702) and *Haemophilus influenza.* Hemophilus influenza type B (HIB) (See, e.g., Costantino et al. (1999) *Vaccine* 17:1251-1263), *Porphyromonas gingivalis* (Ross et al. (2001) *Vaccine* 19:4135-4132) and combinations thereof.

Examples of antigens from Neisseria Meningitides A, B and C are disclosed in the following co-owned patent applications: PCT/US99/09346; PCT IB98/01665; PCT IB99/00103; WO 00/66791; WO 99/24578; WO 00/71574; WO 99/36544; WO 01/04316; WO 99/57280; WO 01/31019; WO 00/22430; WO 00/66741; WO 00/71725; WO 01/37863; WO 01/38350; WO 01/52885; WO 01/64922; WO 01/64920; WO 96/29412; and WO 00/50075.

The complete genomic sequence of MenB, strain MC58, has been described. Tettelin et al., *Science* (2000) 287:1809. Several proteins that elicited serum bactericidal antibody responses have been identified by whole genome sequencing. For example, immunogenic compositions can include an outer-membrane vesicle (OMV) preparation from *N. meningitidis* serogroup B, such as those disclosed in Bjune et al. (1991) *Lancet* 338:1093-1096; Fukasawa et al. (1999) *Vaccine* 17:2951-2958; Rosenqvist et al. (1998) *Dev. Biol. Stand.* 92:323-333) or a saccharide antigen *N. meningitidis* serogroup A, C, W135 and/or Y (*See, e.g.,* Costantino et al. (1992) *Vaccine* 10:691-698; Costantino et al. (1992) *Vaccine* 10:1251-1263. Many proteins from these pathogens have conserved sequences and appear to be surface-exposed on encapsulated

MenB strains. Pizza et al., *Science* (2000) 287:1816. One of these proteins is GNA33 (genome derived antigen). GNA33 is a lipoprotein and the predicted amino acid sequence shows homology with a membrane-bound lytic murein transglycosylase (MltA) from *E. coli* and *Synechocystis* sp. Lommatzsch et al., *J. Bacteriol.* (1997) 179:5465-5470. GNA33 is highly conserved among *Neisseria meningitidis*. Pizza et al., *Science* (2000) 287:1816. Mice immunized with recombinant GNA33 developed high serum bactericidal antibody titers measured against encapsulated MenB strain 2996. The magnitude of the antibody response was similar to that of control animals immunized with OMP vesicles prepared from strain 2996. However, the mechanism by which GNA33 elicits protective antibody was not identified, nor was the breadth of the protective response to different MenB strains.

In certain embodiments, one or more antigens derived from a capsular saccharide are used. Non-limiting examples of such suitable saccharide antigens include those derived from *S.pneumoniae, H.influenzae* and *N.meningitidis*. MenC oligosaccharide antigens conjugated to carrier proteins are described, for example, in U.S. Patent No. 6,251,401; International Publications WO 00/71725 and WO 01/37863. Saccharide antigens from these and other pathogens are known, as is the preparation of polysaccharide conjugates in general. The saccharide moiety of the conjugate may be a polysaccharide (*e.g.* full-length polyribosylribitol phosphate (PRP)) or hydrolysed polysaccharides (*e.g.* by acid hydrolysis) in order to form oligosaccharides (*e.g.* MW from ~1 to ~5 kDa). If hydrolysis is performed, the hydrolysate may be sorted by size in order to remove oligosaccharides that are too short to be usefully immunogenic. Size-separated oligosaccharides are preferred saccharide antigens. Conjugation of saccharides to carriers such as CRM is described, for example, in Costantino et al. (1992) Vaccine 10:691-698

It is to be understood that antigens derived from more than one pathogen and/or more than one serotype of a particular bacterium can be used in the preparation of immunogenic compositions. Prevnar™, for example, includes seven antigens (4, 6B, 9V, 14, 18C, 19F and 23F) derived from approximately 23 serotypes of *S. pneumoniae*.

2. Antigens derived from Viruses

Non-limiting examples of viruses that may be transmitted via mucosal surfaces include meningitis, rhinovirus, influenza, respiratory syncytial virus (RSV), parainfluenza virus (PIV), and the like. For example, the present invention will find use for stimulating an immune response against a wide variety of proteins from the herpesvirus family, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1 and HSV-2 glycoproteins gB, gD and gH; antigens derived from varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and antigens derived from other human herpesviruses such as HHV6 and HHV7. (See, e.g. Chee et al., *Cytomegaloviruses* (J.K. McDougall, ed., Springer-Verlag 1990) pp. 125-169, for a review of the protein coding content of cytomegalovirus; McGeoch et al., *J Gen. Virol.* (1988) 69:1531-1574, for a discussion of the various HSV-1 encoded proteins; U.S. Patent No. 5,171,568 for a discussion of HSV-1 and HSV-2 gB and gD proteins and the genes encoding therefor; Baer et al., *Nature* (1984) 310:207-211, for the identification of protein coding sequences in an EBV genome; and Davison and Scott, *J Gen. Virol.* (1986) 67:1759-1816, for a review of VZV.)

Antigens from the hepatitis family of viruses, including hepatitis A virus (HAV) (*See, e.g.,* Bell et al. (2000) *Pediatr Infect Dis. J.* 19:1187-1188; Iwarson (1995) *APMIS* 103:321-326), hepatitis B virus (HBV) (*See, e.g.,* Gerlich et al. (1990) *Vaccine* 8 Suppl:S63-68 & 79-80), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV), can also be conveniently used in the techniques described herein. By way of example, the viral genomic sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. The HCV genome encodes several viral proteins, including El (also known as E) and E2 (also known as E2/NSI) and an N-terminal nucleocapsid protein (termed "core") (see, Houghton et al., Hepatology (1991) 14:381-388, for a discussion of HCV proteins, including El and E2). Each of these proteins, as well as antigenic fragments thereof and/or nucleic acids encoding the proteins, will find use in the present invention.

Similarly, the sequence for the δ-antigen from HDV is known (see, e.g., U.S. Patent No. 5,378,814) and this antigen can also be conveniently used in the present

invention. Additionally, antigens derived from HBV, such as the core antigen, the surface antigen, sAg, as well as the presurface sequences, pre-S1 and pre-S2 (formerly called pre-S), as well as combinations of the above, such as sAg/pre-S1, sAg/pre-S2, sAg/pre-SI/pre-S2, andpre-SI/pre-S2,will find use herein. See, *e.g.*, "HBV Vaccines-from the laboratory to license: a case study" in Mackett, M. and Williamson, J.D., Human Vaccines and Vaccination, pp. 159-176, for a discussion of HBV structure; and U.S. Patent Nos. 4,722,840, 5,098,704, 5,324,513; Beames et al., *J. Virol.* (1995) 69:6833-6838, Birnbaum et al., *J Virol.* (1990) 64:3319-3330; and Zhou et al., *J Virol.* (1991) 65:5457-5464.

More particularly, the gp120 envelope proteins from any of the above HIV isolates, including members of the various genetic subtypes of HIV, are known and reported (see, e.g., Myers et al., Los Alamos Database, Los Alamos National Laboratory, Los Alamos, New Mexico (1992); Myers et al., Human Retroviruses and,4ids, 1990, Los Alatiios, New Mexico: Los Alamos National Laboratory; and Modrow et al., J Virol. (I 987) 61:570-578, for a comparison of the envelope sequences of a variety of HIV isolates) and antigens derived from any of these isolates will find use in the present methods. Furthermore, the invention is equally applicable to other immunogenic proteins derived from any of the various HIV isolates, including any of the various envelope proteins such as gp160 and gp4l, gag antigens such as p24gag and p55gag, as well as proteins derived from the pol region.

In addition, due to the large immunological variability that is found in different geographic regions for the open reading frame of HIV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, at least eight different subtypes of HIV have been identified and, of these, subtype B viruses are more prevalent in North America, Latin America and the Caribbean, Europe, Japan and Australia. Almost every subtype is present in sub-Saharan Africa, with subtypes A and D predominating in central and eastern Africa, and subtype C in southern Africa. Subtype C is also prevalent in India and it has been recently identified in southern Brazil. Subtype E was initially identified in Thailand, and is also present in the Central African Republic. Subtype F was initially described in Brazil and in Romania. The most recent subtypes described are G, found in Russia and Gabon, and subtype H, found in Zaire and in Cameroon. Group O viruses have been identified in

Cameroon and also in Gabon. Thus, as will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration that is appropriate to the particular HIV subtype that is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, by sequencing the HIV genome (or fragments thereof) isolated from individuals within that region.

As described above, also presented by HIV are various *Gag* and *Env* antigens. HIV-1 *Gag* proteins are involved in many stages of the life cycle of the virus including, assembly, virion maturation after particle release, and early post-entry steps in virus replication. The roles of HIV-1 *Gag* proteins are numerous and complex (Freed, E.O. (1998) *Virology* 251:1-15).

Env coding sequences of the present invention include, but are not limited to, polynucleotide sequences encoding the following HIV-encoded polypeptides: gp160, gp140, and gp120 (see, e.g., U.S. Patent No. 5,792,459 for a description of the HIV-1_{SF2} ("SF2") Env polypeptide). The envelope protein of HIV-1 is a glycoprotein of about 160 kD (gp160). During virus infection of the host cell, gp160 is cleaved by host cell proteases to form gp120 and the integral membrane protein, gp41. The gp41 portion is anchored in (and spans) the membrane bilayer of virion, while the gp120 segment protrudes into the surrounding environment. As there is no covalent attachment between gp120 and gp41, free gp120 is released from the surface of virions and infected cells. Thus, gp160 includes the coding sequences for gp120 and gp41. The polypeptide gp41 is comprised of several domains including an oligomerization domain (OD) and a transmembrane spanning domain (TM). In the native envelope, the oligomerization domain is required for the non-covalent association of three gp41 polypeptides to form a trimeric structure: through non-covalent interactions with the gp41 trimer (and itself), the gp120 polypeptides are also organized in a trimeric structure. A cleavage site (or cleavage sites) exists approximately between the polypeptide sequences for gp120 and the polypeptide sequences corresponding to gp41. This cleavage site(s) can be mutated to prevent cleavage at the site. The resulting gp140 polypeptide corresponds to a truncated form of gp160 where the transmembrane spanning domain of gp41 has been deleted. This gp140 polypeptide can exist in both monomeric and oligomeric (*i.e.* trimeric) forms by virtue of the presence of the oligomerization domain in the gp41

moiety and oligomeric form may be designed "o," for example "ogp140" refers to oligomeric gp140. In the situation where the cleavage site has been mutated to prevent cleavage and the transmembrane portion of gp41 has been deleted the resulting polypeptide product can be designated "mutated" gp140. As will be apparent to those in the field, the cleavage site can be mutated in a variety of ways. (See, also, WO 00/39302).

Influenza virus is another example of a virus for which the present invention will be particularly useful. Specifically, the envelope glycoproteins HA and NA of influenza A are of particular interest for generating an immune response. Numerous HA subtypes of influenza A have been identified (Kawaoka et al., *Virology* (1990) 179:759-767; Webster et al., "Antigenic variation among type A influenza viruses," p. 127-168. In: P. Palese and D.W. Kingsbury (ed.), Genetics of influenza viruses. Springer-Verlag, New York). Thus, proteins derived from any of these isolates can also be used in the compositions and methods described herein.

Antigens derived from other viruses will also find use in the present invention, such as without limitation, proteins from members of the families Picomaviridae (e.g., polioviruses, etc. as described, for example, in Sutter et al. (2000) *Pediatr Clin North Am* 47:287-308; Zimmerman & Spann (1999) *Am Fam Physician* 59:113-118; 125-126); Caliciviridae; Togaviridae (e.g., rubella virus, dengue virus, etc.); the family Flaviviridae, including the genera flavivirus (*e.g.,* yellow fever virus, Japanese encephalitis virus, serotypes of Dengue virus, tick borne encephalitis virus, West Nile virus); pestivirus (*e.g.,* classical porcine fever virus, bovine viral diarrhea virus, border disease virus); and hepacivirus (*e.g.,* hepatitis A, B and C as described, for example, in U.S. Patent Nos. 4,702,909; 5,011,915; 5,698,390; 6,027,729; and 6,297,048); Parvovirsus (*e.g.,* parvovirus B19); Coronaviridae; Reoviridae; Bimaviridae; Rhabodoviridae (e.g., rabies virus, etc. as described for example in Dressen et al. (1997) *Vaccine* 15 Suppl:s2-6; MMWR Morb Mortal Wkly Rep. 1998 Jan 16:47(1):12, 19); Filoviridae; Paramyxoviridae (e.g., mumps virus, measles virus, rubella, respiratory syncytial virus, etc. as described in Chapters 9 to 11 of *Vaccines*, 1998, eds. Plotkin & Mortimer (ISBN 0-7216-1946-0); Orthomyxoviridae (e.g., influenza virus types A, B and C, etc. as described in Chapter 19 of *Vaccines*, 1998, eds. Plotkin & Mortimer (ISBN 0-7216-1946-0),.); Bunyaviridae; Arenaviridae; Retroviradae (e.g., HTLV-1;

HTLV-11; HIV-1 (also known as HTLV-III, LAV, ARV, HTI,R, etc.)), including but not limited to antigens from the isolates HIVIIIb, HIVSF2, HIVLAV, HIVI-AL, I-IIVMN); HIV- I CM235, HIV- I IJS4; HIV-2; simian immunodeficiency virus (SIV) among others. Additionally, antigens may also be derived from human papilloma virus (HPV) and the tick-borne encephalitis viruses. See, e.g. Virology, 3rd Edition (W.K. Joklik ed. 1988); Fundamental Virology, 2nd Edition (B.N. Fields and D.M. Knipe, eds, 1991), for a description of these and other viruses.

In certain embodiments, one or more of the antigens are derived from HIV. The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins divided into three major classes: (1) the major structural proteins, Gag, Pol, and Env; (2) the regulatory proteins, Tat and Rev and (3) the accessory proteins, Vpu, Vpr, Vif, and Nef. Although exemplified herein with relation to antigens obtained from HIV_{SF2}, sequence obtained from other HIV variants may be manipulated in similar fashion following the teachings of the present specification. Such other variants include, but are not limited to, Gag protein encoding sequences obtained from the isolates HIV_{IIIb}, HIV_{SF2}, $HIV\text{-}1_{SF162}$, $HIV\text{-}1_{SF170}$, HIV_{LAV}, HIV_{LAI}, HIV_{MN}, $HIV\text{-}1_{CM235}$, $HIV\text{-}1_{US4}$, other HIV-1 strains from diverse subtypes (e.g., subtypes, A through G, and O), HIV-2 strains and diverse subtypes (e.g., $HIV\text{-}2_{UC1}$ and $HIV\text{-}2_{UC2}$), and simian immunodeficiency virus (SIV). (See, e.g., Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991); *Virology*, 3rd Edition (Fields, BN, DM Knipe, PM Howley, Editors, 1996, Lippincott-Raven, Philadelphia, PA; for a description of these and other related viruses).

Examples of parasitic antigens include those derived from organisms causing malaria and Lyme disease.

3. Tumor Antigens

A variety to tumor antigens have been identified. See, *e.g.*, Moingeon, *supra* and Rosenberg, *supra*. Non-limiting examples of tumor antigens include antigens recognized by CD8+ lymphocytes (*e.g.*, melanoma-melanocyte differentiation antigens such as MART-1, gp100, tyrosinase, tyrosinase related protein-1, tyrosinase related protein-2, melanocyte-stimulating hormone receptor; mutated antigens such as beta-catenin, MUM-1, CDK-4, caspase-8, KIA 0205, HLA-A2-R1701; cancer-testes

antigens such as MAGE-1, MAGE-2, MAGE-3, MAGE-12, BAGE, GAGE and NY-ESO-1; and non-mutated shared antigens over expressed on cancer such as alpha-fetoprotein, telomerase catalytic protein, G-250, MUC-1, carcinoembryonic antigen, p53, Her-2-neu) as well as antigens recognized by CD4+ lymphocytes (*e.g.*, gp100, MAGE-1, MAGE-3, tyrosinase, NY-ESO-1, triosephosphate isomerase, CDC-27, and LDLR-FUT). See, also, WO 91/02062, U.S. Patent No. 6,015,567, WO 01/08636, WO 96/30514, U.S. Patent No. 5,846,538 and U.S. Patent No. 5,869,445.

In certain embodiments, the tumor antigen(s) are derived from mutated or altered cellular components. After alteration, the cellular components no longer perform their regulatory functions, and hence the cell may experience uncontrolled growth. Representative examples of altered cellular components include ras, p53, Rb, altered protein encoded by the Wilms' tumor gene, ubiquitin, mucin, protein encoded by the DCC, APC, and MCC genes, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, platelet derived growth factor (PDGF) receptor, insulin receptor, epidermal growth factor (EGF) receptor, and the colony stimulating factor (CSF) receptor. These as well as other cellular components are described for example in U.S. Patent No. 5,693,522 and references cited therein.

4. Polypeptide Preparation

The antigens in the immunogenic compositions will typically be in the form of proteins. As an alternative to protein-based vaccination, the antigens in the immunogenic compositions may be in the form of nucleic acid molecules or polynucleotides.

Thus, polypeptide antigens can be constructed by solid phase protein synthesis. If desired, the polypeptides also can contain other amino acid sequences, such as amino acid linkers or signal sequences, as well as ligands useful in protein purification, such as glutathione-S-transferase and staphylococcal protein A. Alternatively, antigens of interest can be purchased from commercial sources.

Polypeptides can also be produced from nucleic acids encoding the desired polypeptide. Sequences encoding the polypeptide of interest can be generated by the polymerase chain reaction (PCR). Mullis et al. (1987) *Methods Enzymol.* 155:335-350; *PCR Protocols, A Guide to Methods and Applications*, Innis et al (eds) Harcourt Brace

Jovanovich Publishers, NY (1994)). This technique uses DNA polymerase, usually a thermostable DNA polymerase, to replicate a desired region of DNA. The region of DNA to be replicated is identified by oligonucleotides of specified sequence complementary to opposite ends and opposite strands of the desired DNA to prime the replication reaction. Repeated successive cycles of replication result in amplification of the DNA fragment delimited by the primer pair used. A number of parameters influence the success of a reaction. Among them are annealing temperature and time, extension time, Mg^{2+} and ATP concentration, pH, and the relative concentration of primers, templates, and deoxyribonucleotides.

Once coding sequences for desired proteins have been prepared or isolated, such sequences can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Ligations to other sequences are performed using standard procedures, known in the art.

Similarly, the selected coding sequences can be cloned into any suitable expression vector for expression. The expressed product can optionally be purified prior to mucosal administration. Briefly, a polynucleotide encoding these proteins can be introduced into an expression vector that can be expressed in a suitable expression system. A variety of bacterial, yeast, mammalian, insect and plant expression systems are available in the art and any such expression system can be used. Optionally, a polynucleotide encoding these proteins can be translated in a cell-free translation system. Such methods are well known in the art.

B. DELIVERY

The compositions (*e.g.*, polynucleotides and/or polypeptides) described herein can be delivered using any suitable means (*e.g.*, intravenously, intramuscularly, intraperitoneally, subcutaneously, transcutaneously for parenteral priming and orally, rectally, intraocularly, or intranasally for mucosal boosting), or by various physical methods such as lipofection (Felgner et al. (1989) *Proc. Natl. Acad. Sci. USA* 84:7413-7417), direct DNA injection (Acsadi et al. (1991) *Nature* 352:815-818); microprojectile bombardment (Williams et al. (1991) *PNAS* 88:2726-2730); liposomes of several types (*see, e.g.,* Wang et al. (1987) *PNAS* 84:7851-7855); $CaPO_4$ (Dubensky et al. (1984)

PNAS 81:7529-7533); DNA ligand (Wu et al (1989) *J. of Biol. Chem.* 264:16985-16987); administration of polypeptides alone; administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al. (1992), *Hum. Gene Ther.* 3:147-154); via polycation compounds such as polylysine, utilizing receptor specific ligands; as well as with psoralen inactivated viruses such as Sendai or Adenovirus. Transcutaneous administration may include the use of a penetration enhancer, a barrier disruption agent or combinations thereof. See, *e.g.*. WO 99/43350. In addition, the administration may either be administered directly (*i.e.*, *in vivo*), or to cells that have been removed (*ex vivo*), and subsequently returned.

In a preferred embodiment, the invention provides a method for raising an immune response in a mammal by parenterally administering at least one first immunogenic composition and subsequently administering at least one second immunogenic composition mucosally. In other words, the invention includes a parenteral prime followed by a mucosal boost.

Methods of parenteral administration of polynucleotides and/or polypeptides are well known and include, for example, (1) direct injection into the blood stream (*e.g.*, intravenous administration); (2) direct injection into a specific tissue or tumor; (3) subcutaneous administration; (4) transcutaneous epidermal administration; (5) intradermal administration; (6) intraperitoneal administration; and/or (7) intramuscular administration. Other modes of parenteral administration include pulmonary administration, suppositories, needle-less injection, transcutaneous and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. As noted above, administration of nucleic acids may also be combined with administration of peptides or other substances.

Similarly, methods of mucosal delivery are known in the art, for example as described in *Remington's, supra* and includes nasal, rectal, oral and vaginal delivery. Delivery of the compositions rectally and vaginally is particularly preferred in the case of sexually transmitted pathogens, as this mode of administration provides access to the cells first exposed to the pathogens. Similarly, intranasal administration may be preferred in diseases, like rhinovirus, that infect through nasal mucosa. In some instances, intranasal administration may induce immunity in the vaginal mucosa and oral immunization may induce immunity in the rectal mucosa. Moreover, combinations

of various routes of mucosal administration and/or various routes of systemic administration can be used in order to induce optimal immunity and protection (both at the site the pathogen enters as well as at systemic sites where a mucosal pathogen has spread to. Additionally, mucosal administration eliminates the need for syringes or other administration devices. Dosage treatment may be a single dose schedule or a multiple dose schedule.

The compositions disclosed herein can be administered alone or can be administered with one or more additional macromolecules (*e.g.*, gene delivery vehicles, immunomodulatory factors, adjuvants, and/or one or more proteins). In such embodiments, the multiple compositions can be administered in any order, for example gene delivery vehicle followed by protein; multiple gene delivery vehicles followed by multiple protein administrations; protein administration(s) followed by single or multiple gene delivery vehicle administration; concurrent administration; and the like. Thus, a mixture of protein and nucleic acid can be administered, using the same or different vehicles and the same or different modes of administration.

The interval between priming and boosting will vary according to factors such as the age of the patient and the nature of the composition and these factors can be assessed by a physician. Administration of the first priming and boosting doses is generally separated by at least 2 weeks, typically at least 4 weeks. The methods of the invention may comprise more than one parenteral priming dose and/or more than one boosting dose, e.g., two or more priming doses followed by two or more mucosal booster doses. (see, Example 4 below, describing a "memory" boost 18 months after the initial prime-boost). The term "memory" boost refers to any boosting dose given after the initial boost. The time at which the "memory" boost is administered can vary from hours (*e.g.*, 1 to 72 hours or any timepoint therebetween) or days (*e.g.* 1 to 90 days or any timepoint therebetween) to months (*e.g.*, 1 to 36 months or any timepoint therebetween) or even years after the initial boost. More than one memory boost may be administered at the same or varying time intervals with respect to each other. Identical or different immunogenic compositions may be used for each priming dose. Priming and boosting doses may be therefore distinguished by the route of administration, rather than by their timing.

The mammal to whom the compositions are administered is typically primate, such as a human. The human may be a child or an adult. Suitable lower mammals may include mice.

In certain embodiments, direct delivery will generally be accomplished with or without viral vectors, as described above, by injection using either a conventional syringe or a gene gun, such as the Accell® gene delivery system (PowderJect Technologies, Inc., Oxford, England).

1. Microparticles

In certain embodiments, one or more of the selected antigens are entrapped in, or adsorbed to, a microparticle for subsequent delivery. Biodegradable polymers for manufacturing microparticles useful in the present invention are readily commercially available from, e.g., Boehringer Ingelheim, Germany and Birmingham Polymers, Inc., Birmingham, AL. For example, useful polymers for forming the microparticles herein include those derived from polyhydroxybutyric acid; polycaprolactone; polyorthoester; polyanhydride; as well as a poly(α-hydroxy acid), such as poly(L-lactide), poly(D,L-lactide) (both known as "PLA" herein), poly(hydoxybutyrate), copolymers of D,L-lactide and glycolide, such as poly(D,L-lactide-co-glycolide) (designated as "PLG" or "PLGA" herein) or a copolymer of D,L-lactide and caprolactone. Particularly preferred polymers for use herein are PLA and PLG polymers. These polymers are available in a variety of molecular weights, and the appropriate molecular weight for a given antigen is readily determined by one of skill in the art. Thus, e.g., for PLA, a suitable molecular weight will be on the order of about 2000 to 250,000. For PLG, suitable molecular weights will generally range from about 10,000 to about 200,000, preferably about 15,000 to about 150,000, and most preferably about 50,000 to about 100,000.

If a copolymer such as PLG is used to form the microparticles, a variety of lactide:glycolide ratios will find use herein and the ratio is largely a matter of choice, depending in part on the co administered antigen and the rate of degradation desired. For example, a 50:50 PLG polymer, containing 50% D,L-lactide and 50% glycolide, will provide a fast resorbing copolymer while 75:25 PLG degrades more slowly, and

85:15 and 90:10, even more slowly, due to the increased lactide component. It is readily apparent that a suitable ratio of lactide:glycolide is easily determined by one of skill in the art based on the nature of the antigen and disorder in question. Moreover, mixtures of microparticles with varying lactide:glycolide ratios will find use in the formulations in order to achieve the desired release kinetics for a given antigen and to provide for both a primary and secondary immune response. Degradation rate of the microparticles of the present invention can also be controlled by such factors as polymer molecular weight and polymer crystallinity. PLG copolymers with varying lactide:glycolide ratios and molecular weights are readily available commercially from a number of sources including from Boehringer Ingelheim, Germany and Birmingham Polymers, Inc., Birmingham, AL. These polymers can also be synthesized by simple polycondensation of the lactic acid component using techniques well known in the art, such as described in Tabata et al., *J. Biomed. Mater. Res.* (1988) 22:837-858.

The antigen/microparticles are prepared using any of several methods well known in the art. For example, double emulsion/solvent evaporation techniques, such as described in U.S. Patent No. 3,523,907 and Ogawa et al., *Chem. Pharm. Bull.* (1988) 36:1095-1103, can be used herein to form the microparticles. These techniques involve the formation of a primary emulsion consisting of droplets of polymer solution containing the antigen (if antigen is to be entrapped in the microparticle), which is subsequently mixed with a continuous aqueous phase containing a particle stabilizer/surfactant.

More particularly, a water-in-oil-in-water (w/o/w) solvent evaporation system can be used to form the microparticles, as described by O'Hagan et al., *Vaccine* (1993) 11:965-969; Jeffery et al., Pharm. Res. (1993) 10:362 and PCT/US99/17308 (WO 00/06133). In this technique, the particular polymer is combined with an organic solvent, such as ethyl acetate, dimethylchloride (also called methylene chloride and dichloromethane), acetonitrile, acetone, chloroform, and the like. The polymer will be provided in about a 2-15%, more preferably about a 4-10% and most preferably, a 6% solution, in organic solvent. An approximately equal amount of an antigen solution, e.g., in water, is added and the polymer/antigen solution emulsified using e.g., an homogenizer. The emulsion is then combined with a larger volume of an aqueous solution of an emulsion stabilizer such as polyvinyl alcohol (PVA) or polyvinyl

pyrrolidone. The emulsion stabilizer is typically provided in about a 2-15% solution, more typically about a 4-10% solution. The mixture is then homogenized to produce a stable w/o/w double emulsion. Organic solvents are then evaporated.

The formulation parameters can be manipulated to allow the preparation of small (<5μm) and large (>30μm) microparticles. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; McGee et al., *J. Microencap.* (1996). For example, reduced agitation results in larger microparticles, as does an increase in internal phase volume. Small particles are produced by low aqueous phase volumes with high concentrations of PVA.

Microparticles can also be formed using spray-drying and coacervation as described in, e.g., Thomasin et al., *J. Controlled Release* (1996) 41:131; U.S. Patent No. 2,800,457; Masters, K. (1976) *Spray Drying* 2nd Ed. Wiley, New York; air-suspension coating techniques, such as pan coating and Wurster coating, as described by Hall et al., (1980) The "Wurster Process" in *Controlled Release Technologies: Methods, Theory, and Applications* (A.F. Kydonieus, ed.), Vol. 2, pp. 133-154 CRC Press, Boca Raton, Florida and Deasy, P.B., *Crit. Rev. Ther. Drug Carrier Syst.* (1988) S(2):99-139; and ionic gelation as described by, e.g., Lim et al., *Science* (1980) 210:908-910.

The above techniques are also applicable to the production of microparticles with adsorbed antigens. In this embodiment, microparticles are formed as described above, however, antigens are mixed with the microparticles following formation.

Particle size can be determined by, e.g., laser light scattering, using for example, a spectrometer incorporating a helium-neon laser. Generally, particle size is determined at room temperature and involves multiple analyses of the sample in question (e.g., 5-10 times) to yield an average value for the particle diameter. Particle size is also readily determined using scanning electron microscopy (SEM).

Prior to use of the microparticles, antigen content is generally determined so that an appropriate amount of the microparticles may be delivered to the subject in order to elicit an adequate immune response.

Antigen content of the microparticles can be determined according to methods known in the art, such as by disrupting the microparticles and extracting the entrapped antigen. For example, microparticles can be dissolved in dimethylchloride and the protein extracted into distilled water, as described in, e.g., Cohen et al., *Pharm. Res.*

(1991) 8:713; Eldridge et al., *Infect. Immun.* (1991) 59:2978; and Eldridge et al., *J. Controlled Release* (1990)11:205. Alternatively, microparticles can be dispersed in 0.1 M NaOH containing 5% (w/v) SDS. The sample is agitated, centrifuged and the supernatant assayed for the antigen of interest using an appropriate assay. See, e.g., O'Hagan et al., *Int. J. Pharm.* (1994) 103:37-45.

One method for adsorbing macromolecules onto prepared microparticles is as follows. Microparticles are rehydrated and dispersed to an essentially monomeric suspension of microparticles using dialyzable anionic or cationic detergents. Useful detergents include, but are not limited to, any of the various N-methylglucamides (known as MEGAs), such as heptanoyl-N-methylglucamide (MEGA-7), octanoyl-N-methylglucamide (MEGA-8), nonanoyl-N-methylglucamide (MEGA-9), and decanoyl-N-methyl-glucamide (MEGA-10); cholic acid; sodium cholate; deoxycholic acid; sodium deoxycholate; taurocholic acid; sodium taurocholate; taurodeoxycholic acid; sodium taurodeoxycholate; 3-[(3-cholamidopropyl)dimethylammonio] -1-propane-sulfonate (CHAPS); 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propane-sulfonate (CHAPSO); N-dodecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate (ZWITTERGENT 3-12); N,N-bis-(3-D-gluconeamidopropyl)-deoxycholamide (DEOXY-BIGCHAP); N-octylglucoside; sucrose monolaurate; glycocholic acid/sodium glycocholate; laurosarcosine (sodium salt); glycodeoxycholic acid/sodium glycodeoxycholate; sodium dodceyl sulfate (SDS); and hexadecyltrimethylammonium bromide (CTAB); dodecyltrimethylammonium bromide; hexadecyltrimethyl-ammonium bromide; tetradecyltrimethylammonium bromide; benzyl dimethyldodecylammonium bromide; benzyl dimethyl-hexadecylammonium chloride; benzyl dimethyltetra-decylammonium bromide. The above detergents are commercially available from e.g., Sigma Chemical Co., St. Louis, MO. Various cationic lipids known in the art can also be used as detergents. See Balasubramaniam et al., 1996, *Gene Ther.*, 3:163-72 and Gao, X., and L. Huang. 1995, *Gene Ther.*, 2:7110-722.

The microparticle/detergent mixture is then physically ground, e.g., using a ceramic mortar and pestle, until a smooth slurry is formed. An appropriate aqueous buffer, such as phosphate buffered saline (PBS) or Tris buffered saline, is then added and the resulting mixture sonicated or homogenized until the microparticles are fully

suspended. The macromolecule of interest is then added to the microparticle suspension and the system dialyzed to remove detergent. The polymer microparticles and detergent system are preferably chosen such that the macromolecule of interest will adsorb to the microparticle surface while still maintaining activity of the macromolecule. The resulting microparticles containing surface adsorbed macromolecule may be washed free of unbound macromolecule and stored as a suspension in an appropriate buffer formulation, or lyophilized with the appropriate excipients, as described further below.

2. Additional Particulate Carriers

In addition to microparticles, the compositions may also be encapsulated, adsorbed to, or associated with, particulate carriers. Such carriers present multiple copies of a selected antigen to the immune system and promote migration, trapping and retention of antigens in local lymph nodes. The particles can be taken up by profession antigen presenting cells such as macrophages and dendritic cells, and/or can enhance antigen presentation through other mechanisms such as stimulation of cytokine release.

In certain embodiments, the compositions are delivered using particulate carriers derived from polymethyl methacrylate polymers. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; McGee JP, et al., *J Microencapsul.* 14(2):197-210, 1997; O'Hagan DT, et al., *Vaccine* 11(2):149-54, 1993.

Furthermore, other particulate systems and polymers can be used for the *in vivo* or *ex vivo* delivery of the gene of interest. For example, polymers such as polylysine, polyarginine, polyornithine, spermine, spermidine, as well as conjugates of these molecules, are useful for transferring a nucleic acid of interest. Similarly, DEAE dextran-mediated transfection, calcium phosphate precipitation or precipitation using other insoluble inorganic salts, such as strontium phosphate, aluminum silicates including bentonite and kaolin, chromic oxide, magnesium silicate, talc, and the like, will find use with the present methods. See, e.g., Felgner, P.L., *Advanced Drug Delivery Reviews* (1990) 5:163-187, for a review of delivery systems useful for gene transfer. Peptoids (Zuckerman, R.N., et al., U.S. Patent No. 5,831,005, issued November 3, 1998) may also be used for delivery of a construct of the present invention.

Additionally, biolistic delivery systems employing particulate carriers such as gold and tungsten, are especially useful for delivering synthetic expression cassettes of the present invention. The particles are coated with the synthetic expression cassette(s) to be delivered and accelerated to high velocity, generally under a reduced atmosphere, using a gun powder discharge from a "gene gun." For a description of such techniques, and apparatuses useful therefore, see, e.g., U.S. Patent Nos. 4,945,050; 5,036,006; 5,100,792; 5,179,022; 5,371,015; and 5,478,744. Also, needle-less injection systems can be used (Davis, H.L., et al, *Vaccine* **12**:1503-1509, 1994; Bioject, Inc., Portland, OR).

3. Liposomal/Lipid Delivery Vehicles

The antigens of interest (or polynucleotides encoding these antigens) can also be delivered using liposomes. For example, packaged as DNA or RNA in liposomes prior to delivery to the subject or to cells derived therefrom. Lipid encapsulation is generally accomplished using liposomes that are able to stably bind or entrap and retain nucleic acid. The ratio of condensed DNA to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight, *Biochim. Biophys. Acta.* (1991) 1097:1-17; Straubinger et al., in *Methods of Enzymology* (1983), Vol. 101, pp. 512-527.

Liposomal preparations for use in the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations, with cationic liposomes particularly preferred. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416); mRNA (Malone et al., *Proc. Natl. Acad. Sci. USA* (1989) 86:6077-6081); and purified transcription factors (Debs et al., *J. Biol. Chem.* (1990) 265:10189-10192), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416). Other commercially available lipids include (DDAB/DOPE) and DOTAP/DOPE (Boerhinger). Other cationic

liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g., Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; PCT Publication No. WO 90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. Cationic microparticles can be prepared from readily available materials using techniques known in the art. See, *e.g.*, co-owned WO 01/136599.

Similarly, anionic and neutral liposomes are readily available, such as, from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphoshatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilammelar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger et al., in METHODS OF IMMUNOLOGY (1983), Vol. 101, pp. 512-527; Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; Papahadjopoulos et al., *Biochim. Biophys. Acta* (1975) 394:483; Wilson et al., *Cell* (1979) 17:77); Deamer and Bangham, *Biochim. Biophys. Acta* (1976) 443:629; Ostro et al., *Biochem. Biophys. Res. Commun.* (1977) 76:836; Fraley et al., *Proc. Natl. Acad. Sci. USA* (1979) 76:3348); Enoch and Strittmatter, *Proc. Natl. Acad. Sci. USA* (1979) 76:145); Fraley et al., *J. Biol. Chem.* (1980) 255:10431; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* (1978) 75:145; and Schaefer-Ridder et al., *Science* (1982) 215:166.

The DNA and/or protein antigen(s) can also be delivered in cochleate lipid compositions similar to those described by Papahadjopoulos et al., *Biochem. Biophys. Acta.* (1975) 394:483-491. See, also, U.S. Patent Nos. 4,663,161 and 4,871,488.

4. Gene Delivery Vehicles

In certain embodiments, one or more antigens as described herein are delivered using one or more gene vectors are administered via nucleic acid immunization or the

like using standard gene delivery protocols. Methods for gene delivery are known in the art. See, e.g., U.S. Patent Nos. 5,399,346; 5,580,859; 5,589,466. The constructs can be delivered (*e.g.*, injected) either subcutaneously, epidermally, intradermally, intramuscularly, intravenous, mucosally (such as nasally, rectally and vaginally), intraperitoneally, orally or combinations thereof.

An exemplary replication-deficient gene delivery vehicle that may be used in the practice of the present invention is any of the alphavirus vectors, described in, for example, co-owned U.S. Patent Nos. 6,342,372; 6,329,201 and International Publication WO 01/92552.

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. Selected sequences can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems have been described (U.S. Patent No. 5,219,740; Miller and Rosman, *BioTechniques* (1989) 7:980-990; Miller, A.D., *Human Gene Therapy* (1990) 1:5-14; Scarpa et al., *Virology* (1991) 180:849-852; Burns et al., *Proc. Natl. Acad. Sci. USA* (1993) 90:8033-8037; and Boris-Lawrie and Temin, *Cur. Opin. Genet. Develop.* (1993) 3:102-109.

A number of adenovirus vectors have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham, *J. Virol.* (1986) 57:267-274; Bett et al., *J. Virol.* (1993) 67:5911-5921; Mittereder et al., *Human Gene Therapy* (1994) 5:717-729; Seth et al., *J. Virol.* (1994) 68:933-940; Barr et al., *Gene Therapy* (1994) 1:51-58; Berkner, K.L. *BioTechniques* (1988) 6:616-629; and Rich et al., *Human Gene Therapy* (1993) 4:461-476).

Additionally, various adeno-associated virus (AAV) vector systems have been developed for gene delivery. AAV vectors can be readily constructed using techniques well known in the art. See, e.g., U.S. Patent Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 (published 23 January 1992) and WO 93/03769 (published 4 March 1993); Lebkowski et al., *Molec. Cell. Biol.* (1988) 8:3988-3996; Vincent et al., *Vaccines 90* (1990) (Cold Spring Harbor Laboratory Press); Carter, B.J.

Current Opinion in Biotechnology (1992) 3:533-539; Muzyczka, N. *Current Topics in Microbiol. and Immunol.* (1992) 158:97-129; Kotin, R.M. *Human Gene Therapy* (1994) 5:793-801; Shelling and Smith, *Gene Therapy* (1994) 1:165-169; and Zhou et al., *J. Exp. Med.* (1994) 179:1867-1875.

Another vector system useful for delivering polynucleotides, mucosally and otherwise, is the enterically administered recombinant poxvirus vaccines described by Small, Jr., P.A., et al. (U.S. Patent No. 5,676,950, issued October 14, 1997) as well as the vaccinia virus and avian poxviruses. By way of example, vaccinia virus recombinants expressing the genes can be constructed as follows. The DNA encoding the particular synthetic Gag/antigen coding sequence is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells that are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the coding sequences of interest into the viral genome. The resulting TK$^-$ recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the genes. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an avipox vector is particularly desirable in human and other mammalian species since members of the avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, e.g., WO 91/12882; WO 89/03429; and WO 92/03545. Picornavirus-derived vectors can also be used. (See, e.g., U.S. Patent Nos. 5,614,413 and 6,063,384).

Molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al., *J. Biol. Chem.* (1993) 268:6866-6869 and Wagner et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:6099-6103, can also be used for gene delivery.

A vaccinia based infection/transfection system can be conveniently used to provide for inducible, transient expression of the coding sequences of interest (for example, a synthetic Gag/HCV-core expression cassette) in a host cell. In this system, cells are first infected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA that is then translated into protein by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation products. See, e.g., Elroy-Stein and Moss, *Proc. Natl. Acad. Sci. USA* (1990) 87:6743-6747; Fuerst et al., *Proc. Natl. Acad. Sci. USA* (1986) 83:8122-8126.

As an alternative approach to infection with vaccinia or avipox virus recombinants, or to the delivery of genes using other viral vectors, an amplification system can be used that will lead to high level expression following introduction into host cells. Specifically, a T7 RNA polymerase promoter preceding the coding region for T7 RNA polymerase can be engineered. Translation of RNA derived from this template will generate T7 RNA polymerase that in turn will transcribe more template. Concomitantly, there will be a cDNA whose expression is under the control of the T7 promoter. Thus, some of the T7 RNA polymerase generated from translation of the amplification template RNA will lead to transcription of the desired gene. Because some T7 RNA polymerase is required to initiate the amplification, T7 RNA polymerase can be introduced into cells along with the template(s) to prime the transcription reaction. The polymerase can be introduced as a protein or on a plasmid encoding the RNA polymerase. For a further discussion of T7 systems and their use for transforming cells, see, e.g., International Publication No. WO 94/26911; Studier and Moffatt, *J. Mol. Biol.* (1986) 189:113-130; Deng and Wolff, *Gene* (1994) 143:245-249; Gao et al., *Biochem. Biophys. Res. Commun.* (1994) 200:1201-1206; Gao and Huang, *Nuc. Acids Res.* (1993) 21:2867-2872; Chen et al., *Nuc. Acids Res.* (1994) 22:2114-2120; and U.S. Patent No. 5,135,855.

D. PHARMACEUTICAL COMPOSITIONS

The present invention also includes pharmaceutical compositions comprising polypeptpide or polynucleotide antigens in combination with a pharmaceutically acceptable carrier, diluent, or recipient. Further, other ingredients, such as adjuvants, may also be present. As described more fully in U.S. Patent No. 6,015,694, storage stable and easy administerable immunogenic compositions are particularly needed in Third World countries where refrigeration and/or traditional administration means (syringes, etc.) are not readily available.

In certain embodiments, the compositions include one or more polypeptides. The preparation of immunogenic compounds that contain immunogenic polypeptide(s) as active ingredients is known to those skilled in the art. Typically, such immunogenic compounds are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified, or the protein encapsulated in liposomes.

Compositions of the invention preferably comprise a pharmaceutically acceptable carrier. The carrier should not itself induce the production of antibodies harmful to the host. Pharmaceutically acceptable carriers are well known to those in the art. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; McGee et al. (1997) *J Microencapsul.* 14(2):197-210; O'Hagan et al. (1993) *Vaccine* 11(2):149-54. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid, such as toxoid from diphtheria, tetanus, cholera, etc., as well as toxins derived from *E. coli*.

Pharmaceutically acceptable salts can also be used in compositions of the invention, for example, mineral salts such as hydrochlorides, hydrobromides, phosphates, or sulfates, as well as salts of organic acids such as acetates, proprionates, malonates, or benzoates. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, and other proteins well known to those of skill in the art. Compositions of the invention can also contain liquids or excipients, such as water, saline, glycerol, dextrose, ethanol, or the like, singly or in combination, as well as substances such as wetting agents, emulsifying agents, or pH buffering agents. Liposomes can also be used as a carrier for a composition of the invention, such liposomes are described above.

Further, the compositions described herein can include various excipients, adjuvants, carriers, auxiliary substances, modulating agents, and the like. Preferably, the compositions will include an amount of the antigen sufficient to mount an immunological response. An appropriate effective amount can be determined by one of skill in the art. Such an amount will fall in a relatively broad range that can be determined through routine trials and will generally be an amount on the order of about 0.1 μg to about 1000 μg, more preferably about 1 μg to about 300 μg, of particle/antigen.

Such adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (International Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL

+ CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes) (see, e.g., International Publication WO 00/00249); (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), beta chemokines (MIP, 1-alpha, 1-beta Rantes, etc.); (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International Publication Nos. W093/13202; W092/19265; WO 95/17211; WO 98/18928 and WO 01/22993); (7) CpG containing oligo, bioadhesive polymers, see WO 99/62546 and WO 00/50078; and (8) other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acteyl-normuramyl-L-alanyl-D-isogluatme (nor-MDP), N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(l'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

Where a saccharide or carbohydrate antigen is used, it may be conjugated to a carrier protein. (See, e.g., U.S. Patent No. 5,306,492; EP 0 477 508; WO 98/42721; Ramsay et al. (2001) *Lancet* 357:195-196; *"Conjugate Vaccines"* eds. Cruse et al., ISBN 3805549326). Preferred carrier proteins include bacterial toxins or toxoids, such as diptheheria (*e.g.*, CRM_{197}) or tetanus toxoids. Other suitable carrier proteins include the *N. meningitidis* outer member protein (EP 0372501); synthetic peptides (EP 0378881 and EP 0427347); heat shock proteins (WO 93/17712); cytokines, lymphokines, hormones, growth factors, pertussis proteins (WO 98/58668; EP 0471177); protein D from *H. influenza* (WO 00/56360); toxin A or B from *C. difficile* (WO 00/61761) and the like. It is possible to use mixtures of carrier proteins. Where a mixture comprises capsular saccharides from both serogroups A and C, it is preferred

that the ratio (w/w) of MenA saccharide:MenC saccharide is greater than 1 (*e.g.*,2:1, 3:1, 4:1, 5:1, 10:1 or higher). Saccharides from different serogroups or different pathogens (*e.g.*,. different serogroups of *N. meningitidis*) may be conjugated to the same or different carrier proteins.

The pharmaceutical compositions may also be lyophilized or otherwise made storage-stable.

Administration of the pharmaceutical compositions described herein may be by any suitable route (see, *e.g.*, above). Particularly preferred is a parenteral prime (or multiple primes) following by a mucosal boost (or multiple mucosal boosts). In addition, the administration may take the form of multiple prime-boost administrations. Thus, dosage treatment may be a single prime/boost dose schedule or a multiple prime/boost dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals, chosen to maintain and/or reinforce the immune response, for example at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the potency of the modality, the vaccine delivery employed, the need of the subject and be dependent on the judgment of the practitioner.

Multiple administrations (*e.g.*, prime-boost type administration) are advantageously employed. For example, recombinant alphavirus particles expressing the antigen(s) of interest are administered (*e.g.*, IVAG or IR). Subsequently, the antigen(s) are administered, for example in compositions comprising the polypeptide antigen(s) and a suitable adjuvant. Alternatively, antigens are administered prior to gene delivery vehicles. Multiple polypeptide and multiple gene delivery vehicle administrations (in any order) may also be employed.

The compositions may preferably comprise a "therapeutically effective amount" of the macromolecule of interest. That is, an amount of macromolecule/microparticle will be included in the compositions that will cause the subject to produce a sufficient response, in order to prevent, reduce, eliminate or diagnose symptoms. The exact amount necessary will vary, depending on the subject being treated; the age and general condition of the subject to be treated; the severity of the condition being treated; in the case of an immunological response, the capacity of the subject's immune system to

synthesize antibodies; the degree of protection desired and the particular antigen selected and its mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. Thus, a " therapeutically effective amount" will fall in a relatively broad range that can be determined through routine trials. For example, for purposes of the present invention, where the macromolecule is a polynucleotide, an effective dose will typically range from about 1 ng to about 1 mg, more preferably from about 10 ng to about 1 μg, and most preferably about 50 ng to about 500 ng of the macromolecule delivered per dose; where the macromolecule is an antigen, an effective dose will typically range from about 1 μg to about 100 mg, more preferably from about 10 μg to about 1 mg, and most preferably about 50 μg to about 500 μg of the macromolecule delivered per dose.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLE 1

SERUM IGG AND VAGINAL WASH IGA TITERS FOLLOWING PARENTERAL PRIME - MUCOSAL BOOST WITH HIV ANTIGENS

Mice were primed 2 times intramuscularly with gp120 protein adsorbed onto anionic PLG DSS microparticles. 10 micrograms of the gp120/PLG was given at days 0 and 14. The animals were mucosally boosted 3 times at 10-day intervals. The mucosal boosting was intravaginally, intrarectally or intranasally, with mucosal adjuvants of ACP a bioadhesive polymer (Fidia), LTR72 (Chiron S.p.A.) or CpG containing oligos, 1826 H.C. Davis et al., J. Immunology (1998) 160:870-876.

The effect of mucosal boosting after parenteral priming was investigated and results are shown in Table 1.

Table 1

Grp	Route	Prime	Route	Boost	Vaginal Wash IgA titer	Serum IgG titer
1	IMx2	gp120/PLG 10 µg	-	No boost	22 ± 11	15790 ± 7578
2	IMx2	gp120/PLG 10 µg	IVagx3	gp120/ACP 100 ug + LTR72 10 ug	1055 ± 979	38091±18525
3	IMx2	gp120/PLG 10 µg	IRx3	gp120/ACP 100 ug + LTR72 10 ug	7716±8175	420134±269530
4	IMx2	gp120/PLG 10 µg	INx3	gp120 30 ug + LTR72 10 ug + CPG 50 ug	12421±10156	136137±92334

IMx2- two intramuscular administrations
IVagx3 - three intravaginal administrations
IRx3- three intrarectal administrations
INx3 - three intranasal administrations

As is shown in Table 1 and Figure 1, the mucosal IgA titers as determined by a vaginal wash, and serum IgG titers were increased in the animals that were mucosally boosted as compared to those with no mucosal boost.

EXAMPLE 2

SERUM TITERS AFTER PARENTERAL PRIMING AND MUCOSAL BOOSTING WITH HIV ANTIGENS

The following example shows increased serum IgG titer following mucosal boosting after IM priming.

Mice were immunized intramuscularly with 10 micrograms of gp120/PLG, as described in Example 1. Three mucosal (intranasally or intrarectally) boosts were given with mucosal adjuvants LTR72, ACP or CpG (1826), as described above.

Table 2

Proj. #99-01414						
Grp	route	Prime	route	Boost	Post prime Serum IgG titer	Post Boost Serum IgG titer
					Mean (±SD;N=5)	Mean (±SD;N=5)
1	IMx2	gp120/PLG 10 µg	-	No boost	913 (976)	400 (303)
2	IMx2	gp120/PLG 10 µg	IVagx3	gp120/PLG100 ug + LTR72	505 (393)	1385 (816)
3	IMx2	gp120/PLG 10 µg	IRx3	gp120 100 ug + LTR72	620 (238)	3475 (2322)
5	IMx2	gp120/PLG 10 µg	IRx3	gp120/ACP100 ug + LTR72	555 (429)	6364 (4831)
5	IMx2	gp120/PLG 10 µg	INx3	gp120 30 ug + LTR72 + CPG 50 ug	587 (565)	2662 (2382)

IMx2- two intramuscular administrations; IVagx3 - three intravaginal administrations; IRx3- three intrarectal administrations; INx3 - three intranasal administrations

Table 2 shows that mean serum IgG titer is increased for those animals receiving the mucosal boost.

EXAMPLE 3

VAGINAL WASH IGA TITERS AFTER PARENTERAL PRIMING AND MUCOSAL BOOSTING

The following example shows increased mucosal (vaginal wash) IgA titer following mucosal boosting after IM priming. Mice were immunized as described in Examples 1 and 2. Results are shown in Table 3.

Table 3

Grp	Route	Prime	Route	Boost	Animal #	**Normalized Titers**
1	IMx2	gp120/PLG 10 µg	-	No boost	1	27
					2	10
					3	<10
					4	40
					5	27
					6	21
					7	39
					8	<10
					9	21
					10	25
9	IMx2	gp120/PLG 10 µg	IVagx3	gp120/ACP 100 ug	81	2,128
				+ LTR72 10 ug	82	1,465
					83	1,939
					84	260
					85	34
					86	16
					87	1,662
					88	2,716
					89	52
					90	279
10	IMx2	gp120/PLG 10 µg	IRx3	gp120/ACP 100 ug	91	3,068
				+ LTR72 10 ug	92	H
					93	2,976
					94	1,909
					95	5,260
					96	23,528
					97	19,137
					98	888
					99	16,853
					100	473
11	IMx2	gp120/PLG 10 µg	INx3	gp120 30 ug +	101	4,133
				LTR72 10 ug + CPG 50 ug	102	7,929
					103	1,691
					104	H
					105	27,872
					106	2,517
					107	25,121
					108	6,825
					109	5,183
					110	15,070

The results shown in Table 3 demonstrate that mucosal titers, as measured by vaginal wash IgA titers, are increased following parenteral polypeptide administration and mucosal boosting.

EXAMPLE 4

SERUM TITERS FOLLOWING MEMORY BOOSTING

The following example shows increased serum IgG titers following memory mucosal (intranasal) boosting after parenteral (intramuscular) priming. Mice were immunized essentially as described above except memory boosting was conducted 18 months after the first prime. Results are shown in Table 4 and Figure 2.

Table 4

Grp	Prime/adjuvant	Boost/adjuvant	Memory Boost/ adjuvant	Serum IgG titer
1	IMx2 Ogp140soluble 10 μg / MF59	none	IM Ogp140soluble 10μg / MF59	2037±1897
2	IMx2 Ogp140soluble 10 μg /MF59	INx3 Ogp140/PLG	IN Og140 30 μg/LTR72 10μg + CpG 50 μg	4062±2291
3	IM gp140DNA	INx3 Ogp140 30μg/ LTR72 10μg + CpG 50μg	IN Ogp140 30μg/LTR72 10 μg + CpG 50 μg	7897±4742

IMx2- two intramuscular administrations
IM - one intramuscular administration
IN - one intranasal administration
INx3 - three intranasal administrations

These results demonstrate that serum titers, as measured by ELISA, are increased following mucosal memory boosting at 18 months. Titers are also increased when the parenteral priming is with DNA as compared to protein.

EXAMPLE 5

TITERS FOLLOWING PARENTERAL PRIME - MUCOSAL BOOST WITH *NEISSERIA MENINGITIDIS B* (MENB)-PLG

Mice are primed and boosted with MenB 287 antigen (see, WO 00/66791) as described above. The MenB287 antigen is formulated with PLG microparticles and/or CpG. Results are shown below in Table 5. "IM" refers to intramuscular administration, "IN" refers to intranasal administration. "Imm #" refers to the number of immunizations. Immunization 1 was given on day 0; immunization 2 was given on day 28; immunization 3 was given on day 84; and immunization 4 was given on day 98. "2wp2" refers to titers obtained from bleeds taken 2 weeks after immunization #2 (day 42); "2wp3" refers to titers obtained from bleeds taken 2 weeks after immunization #3 (day 98); and "2wp4" refers to titers obtained from bleeds taken 2 weeks after immunization #4 (day 112).

Table 5

Group	Formulation	Route	Imm #	2wp2	2wp3	2wp4
1	PLG/287 + PLG/CpG, 20 ug	IM	1, 2, 3	15,673	4,163	NA
2	PLG/287, 20 ug	IM	1, 2, 3	10,729	2,853	NA
3	PLG/287 + PLG/CpG, 20 ug	IM	1, 2	34,891	15,167	16,556
	287 + LTK63, 20 ug	IN	3, 4			
4	PLG/287, 20 ug	IM	1, 2	9,064	7,948	9,412
	287 + LTK63, 20 ug	IN	3, 4			

As shown in Table 5, titers are significantly increased when the 3rd immunization is intranasal as compared to intramuscular. Titer also remains elevated (or are increased) following a second mucosal boost (immunization #4).

EXAMPLE 6

SERUM IGG AND VAGINAL WASH IGA TITERS FOLLOWING PARENTERAL PRIME - MUCOSAL BOOST WITH *NEISSERIA MENINGITIDIS* OR *HEMOPHILUS INFLUENZA* (HIB) ANTIGENS

Mice are primed and boosted with MenC or HIB antigens according to the following schedule:

Immunization Schedule

Grp	Day	Route	Vaccine	Adjuvant	Dose of Vaccine
1	0	IN	MenC or HIB	LTK63 or 72	one-fourth the human dose
	14	IN	MenC or HIB	LTK63 or 72	one-fourth the human dose
	28	SC	MenC or HIB	alum	one-fourth the human dose
2	0	SC	MenC or HIB	alum	one-fourth the human dose
	14	IN	MenC or HIB	LTK63 or 72	one-fourth the human dose
	28	IN	MenC or HIB	LTK63 or 72	one-fourth the human dose
3	0	IN	MenC or HIB	LTK63 or 72	one-fourth the human dose
	14	IN	MenC or HIB	LTK63 or 72	one-fourth the human dose
	28	IN	MenC or HIB	LTK63 or 72	one-fourth the human dose
4	0	SC	MenC or HIB	alum	one-fourth the human dose
	14	SC	MenC or HIB	alum	one-fourth the human dose
	28	SC	MenC or HIB	alum	one-fourth the human dose

IN- intranasal administration
SC-subcutaneous administration

For all groups, ELISAs are preformed according to standard procedures before the first dose (i.e. prior to day 0) and after each immunization. For MenC, bactericidal antibody titer assays can also be used to evaluate immune response. Group 2 exhibits greater systemic and/or mucosal immune responses as compared to the other groups.

Claims

What is claimed is:

1. A method of generating an immune response in a subject, comprising

(a) parenterally administering a first immunogenic composition comprising one or more polypeptide antigens and;

(b) mucosally administering a second immunogenic composition comprising one or more antigens, thereby inducing an immune response in the subject.

2. The method of claim 1, wherein the mucosal administration is intranasal.

3. The method of claim 1, wherein the mucosal administration is intrarectal.

4. The method of claim 1, wherein the mucosal administration is intravaginal.

5. The method of claim 1, where in the parenteral administration is transcutaneous.

6. The method of any of claims 1 to 5, wherein the first immunogenic composition further comprises a microparticle.

7. The method of any of claims 1 to 5, wherein the second immunogenic composition is delivered using a microparticle.

8. The method of claim 6 or 7, wherein the microparticle comprises PLG.

9. The method of any of claims 1 to 8, wherein the immune response is a systemic immune response.

10. The method of any of claims 1 to 9, wherein the immune response is a mucosal immune response.

11. The method of any of claims 1 to 10, wherein the immune response is generated to an antigen from one or more pathogens.

12. The method of claim 11, wherein the pathogen is a bacteria.

13. The method of claim 12, wherein the bacteria is *Neisseria meningitidis.*

14. The method of claim 13, wherein the bacteria is *Neisseria meningitidis*, subgroup B.

15. The method of claim 13, wherein the bacteria is *Neisseria meningitidis*, subgroup C.

16. The method of claim 15, wherein the antigens capsular oligosaccharides.

17. The method of claim 16, wherein the saccharides are conjugated to CRM197.

18. The method of claim 12, wherein the bacteria is Haemophilus influenzae type B (HIB).

19. The method of claim 12, wherein the bacteria is *Streptococcus pneumoniae.*

20. The method of claim 12, wherein the bacteria is *Streptococcus agalactiae.*

21. The method of claim 11, wherein the pathogen is a virus.

22. The method of claim 21, wherein the virus is selected from the group consisting of a hepatitis A virus (HAV), human immunodeficiency virus (HIV), respiratory syncytial virus (RSV), parainfluenza virus (PIV), influenza, hepatitis B virus (HBV), herpes simplex virus (HSV), hepatitis C virus (HCV) and human papilloma virus (HPV).

23. The method of claim 22, wherein the virus is HIV-1.

24. The method of claim 22, wherein the virus is RSV.

25. The method of claim 22, wherein the virus is PIV.

26. The method of claim 22, wherein the virus is HCV.

27. The method of any of claims 1 to 10, wherein one or more of the antigens are tumor antigens.

28. The method of any of claims 1 to 27, wherein the first and second immunogenic compositions comprise antigens from the same pathogen.

29. The method of claim 28, wherein the first and second immunogenic compositions are the same.

30. The method of claim 28, wherein the second immunogenic composition comprises at least one antigen that is different than the antigens of the first immunogenic composition.

31. The method of any of claims 1 to 27, wherein the first and second immunogenic compositions comprise antigens from different pathogens.

32. The method of any of claims 1 to 31, wherein the first immunogenic composition further comprises at least one polynucleotide encoding one or more antigens.

33. The method of any of claims 1 to 32, wherein one or more of the antigens of the second immunogenic are encoded by one or more polynucleotides.

34. The method of any of claims 1 to 33, wherein the antigens of the second immunogenic composition comprise polypeptides.

35. The method of any of claims 1 to 34, wherein step (a) is performed two or more times.

36. The method of claim any of claims 1 to 35, wherein step (b) is performed two or more times.

37. A method of generating an immune response against a tumor antigen in a subject comprising

parenterally administering a first immunogenic composition comprising one or more tumor antigens and;

mucosally administering a second immunogenic composition comprising one or more tumor antigens.

38. The method of claim 37, wherein the first immunogenic composition comprises one or more polynucleotides encoding said tumor antigens.

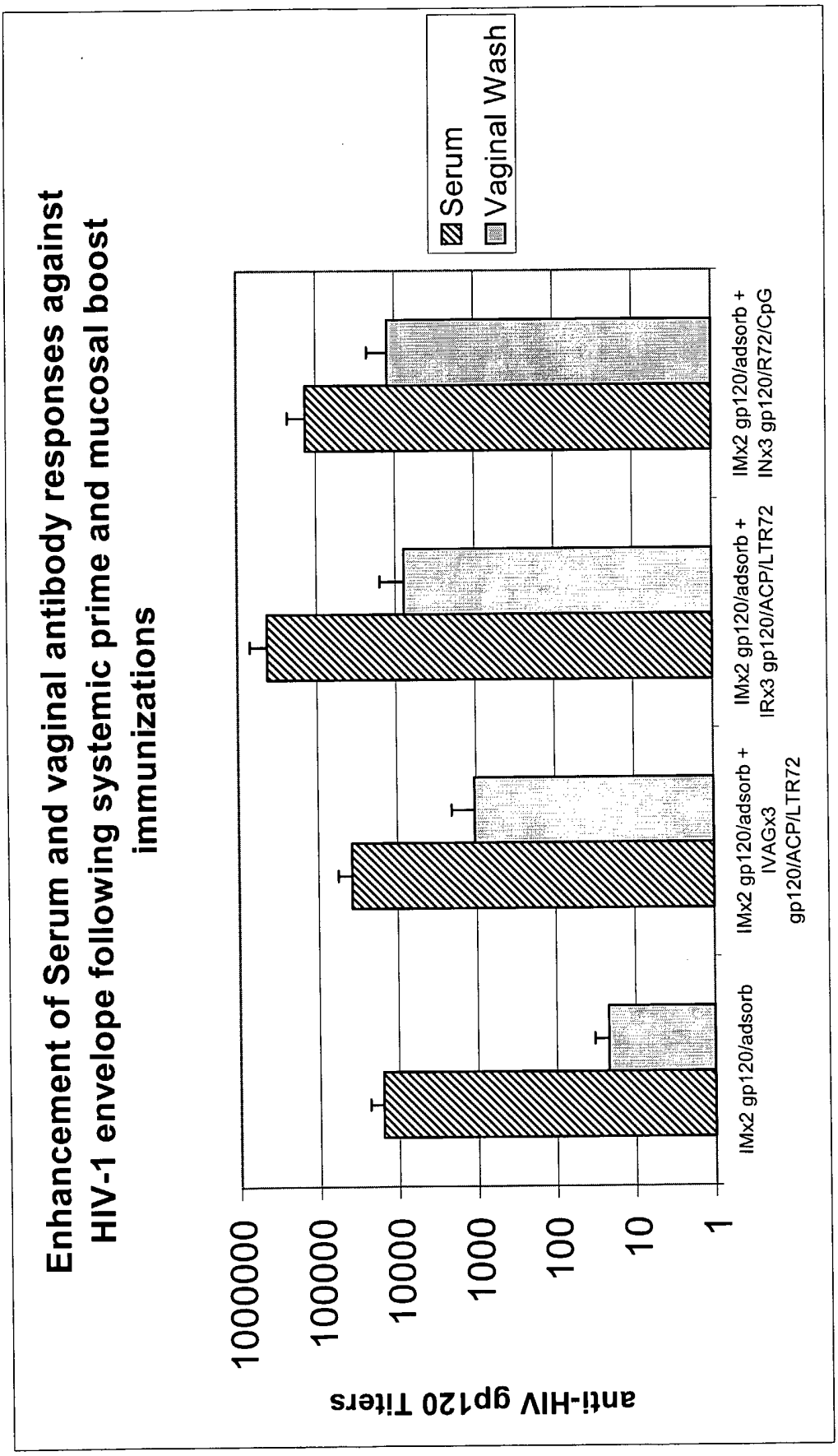
Enhancement of Serum and vaginal antibody responses against HIV-1 envelope following systemic prime and mucosal boost immunizations
Serum
Vaginal Wash
anti-HIV gp120 Titers
1000000
100000
10000
1000
100
10
1
IMx2 gp120/adsorb
IMx2 gp120/adsorb + IVAGx3 gp120/ACP/LTR72
IMx2 gp120/adsorb + IRx3 gp120/ACP/LTR72
IMx2 gp120/adsorb + INx3 gp120/R72/CpG

FIGURE 1

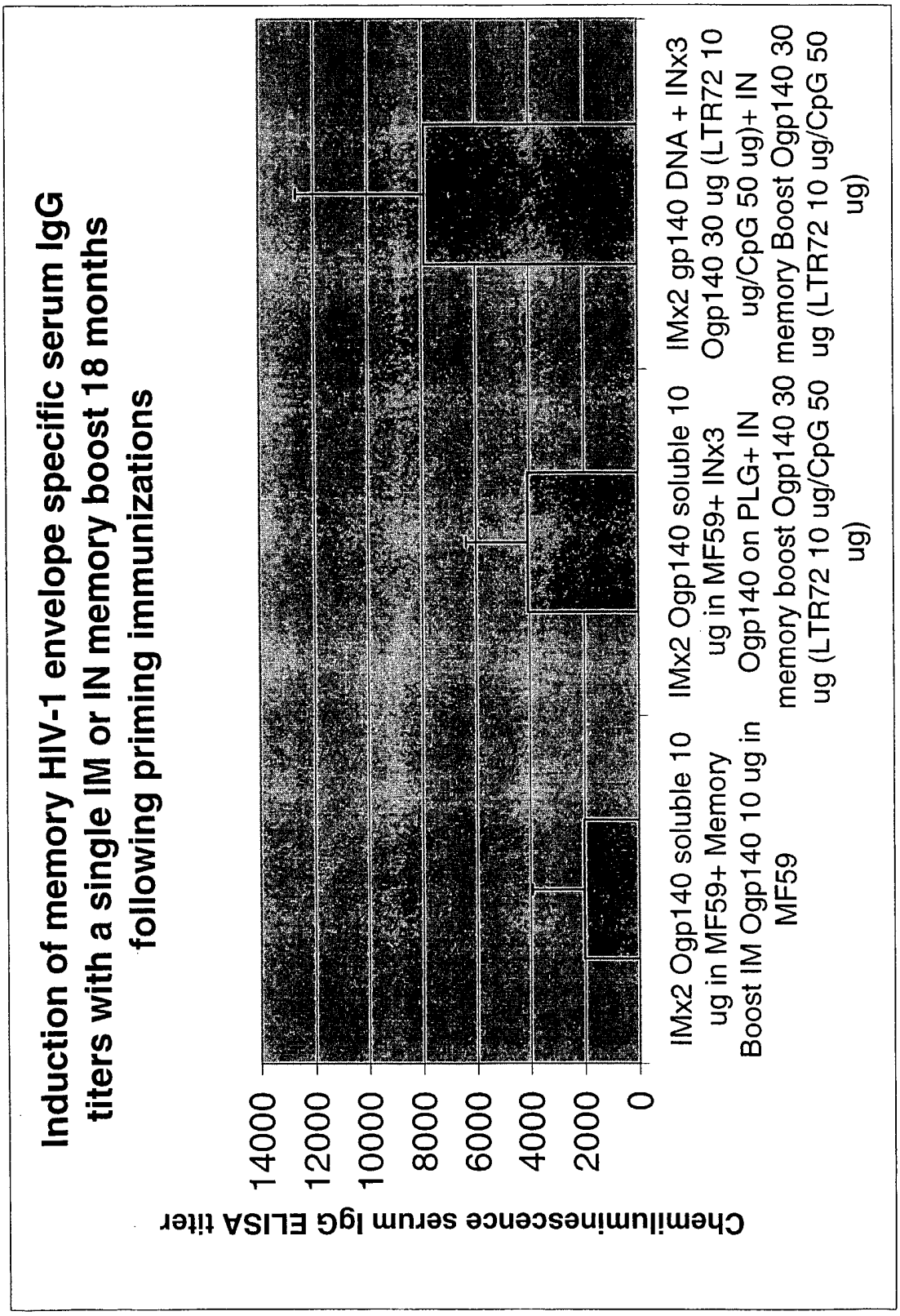
Induction of memory HIV-1 envelope specific serum IgG titers with a single IM or IN memory boost 18 months following priming immunizations
Chemiluminescence serum IgG ELISA titer
14000
12000
10000
8000
6000
4000
2000
0
IMx2 Ogp140 soluble 10 ug in MF59+ Memory Boost IM Ogp140 10 ug in MF59
IMx2 Ogp140 soluble 10 ug in MF59+ INx3
Ogp140 on PLG+ IN memory boost Ogp140 30 ug (LTR72 10 ug/CpG 50 ug)
IMx2 gp140 DNA + INx3 Ogp140 30 ug (LTR72 10 ug/CpG 50 ug)+ IN memory Boost Ogp140 30 ug (LTR72 10 ug/CpG 50 ug)

FIGURE 2