(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 January 2009 (08.01.2009)

PCT

(10) International Publication Number
WO 2009/006479 A2

(51) International Patent Classification:
A61K 48/00 (2006.01)

(21) International Application Number:
PCT/US2008/068924

(22) International Filing Date: 1 July 2008 (01.07.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/947,601 2 July 2007 (02.07.2007) US

(71) Applicant *(for all designated States except US)*: **ETUBICS CORPORATION** [US/US]; 1124 Columbia Steet, Seattle, Washington 98104 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BALINT, Joseph, P.** [US/US]; 520 Second Avenue West, Suite 311, Seattle, Washington 98119 (US). **JONES, Frank, R.** [US/US]; 2041 Kimberly Lane, Ellensburg, Washington 98926 (US). **GAYLE, Richard, B., III** [US/US]; 8209 232nd Street Southeast, Woodinville, Washington 98072 (US).

(74) Agents: **LAHERTY, Carol, D.** et al.; Seed Intellectual Property Law Group PLLC, Suite 5400, 701 Fifth Avenue, Seattle, Washington 98104-7064 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

(54) Title: METHODS AND COMPOSITIONS FOR PRODUCING AN ADENOVIRUS VECTOR FOR USE WITH MULTIPLE VACCINATIONS

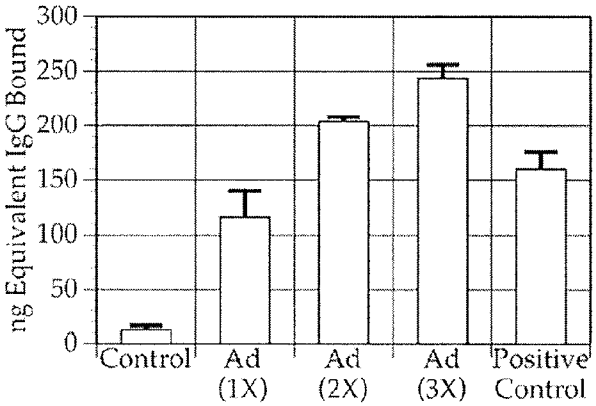

FIG. 1

(57) Abstract: Methods for generating immune responses using adenovirus vectors that allow multiple vaccinations and vaccinations in individuals with preexisting immunity to adenovirus are provided.

WO 2009/006479 A2

METHODS AND COMPOSITIONS FOR PRODUCING AN ADENOVIRUS VECTOR FOR USE WITH MULTIPLE VACCINATIONS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application No. 60/947,601, filed July 2, 2007; where this provisional application is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENT INTEREST

This invention was made with government support under Contract No. 1 R43 AI071733-01 awarded by the National Institutes of Health, National Institutes of Allergy and Infectious Diseases. The government may have certain rights in this invention.

BACKGROUND

Technical Field

The present invention relates to methods for generating immune responses using adenovirus vectors that allow for multiple vaccination regimens.

Description of the Related Art

The most difficult problem with adenovirus vectors has been their inability to sustain long-term transgene expression due largely to the host immune response that eliminates the adenovirus vector and virally transduced cells in immune-competent subjects. Thus, the use of First Generation adenovirus vector vaccines is severely limited by preexisting or induced immunity of vaccines to adenovirus (Ad) (Yang, *et al.* J Virol 77/799-803 (2003); Casimiro, *et al.* J Virol 77/6305-6313 (2003)). One group reported that a preponderance of humans have antibody against adenovirus type 5 (Ad5), the most widely used serotype for gene transfer vectors, and that two-thirds of humans studied have lympho-proliferative

responses against Ad (Chirmule, *et al.* Gene Ther 6/1574-1583 (1999)). In another study, an adenovirus vector vaccine carrying an HIV-1 envelope gene was incapable of reimmunizing a primed immune response using non-adjuvanted DNA (Barouch, *et al.* J. Virol 77/8729-8735 (2003)). Another group reported that non-human primates having pre-existing immunity against Ad5 due to a single immunization with Ad5 were unable to generate transgene-specific antibodies to HIV proteins, as well as altering the overall T cell responses (McCoy, *et al.* J Virol 81/6594-6604 (2007)).

There are numerous mechanisms by which preexisting immunity interferes with adenovirus vector vaccines but the simplest is the presence of neutralizing antibody followed by cell mediated immune elimination of Ad infected antigen harboring cells. Both of these responses are directed to several Ad proteins. Several approaches have been proposed to overcome the barrier of preexisting anti-vector immunity. Perhaps the most straightforward approach would be to increase the vector vaccine dose. Although there is evidence that increasing vaccine doses can increase induction of desired cell mediated immune (CMI) responses in Ad-immune animals (Barouch, *et al.* J. Virol 77/8729-8735 (2003)), it often results in unacceptable adverse effects in animals and humans. Consequently, most investigators using First Generation Ad5 vector vaccines use the approach of a heterologous prime-boost regimen, using naked (non-vectored) DNA as the priming vaccination, followed by an Ad5 vector immunization. This protocol also results in a subsequent immune response against Ad5 such that one cannot administer a further re-immunization (boost) with the same (or a different) adenovirus vector vaccine that utilizes the same viral backbone. Therefore, with the current First Generation of Ad5 vectors, using this approach also abrogates any further use of Ad5 vector immunization in the Ad5 immunized vaccinee.

First Generation (E1 deleted) adenovirus vector vaccines express Ad late genes, albeit at a decreased level and over a longer time period than wild-type Ad virus (Nevins, *et al.* Cell 26/213-220 (1981); Gaynor, *et al.* Cell 33/683-693 (1983); Yang, *et al.* J Virol 70/7209-7212 (1996)). When using First Generation adenovirus vectors for immunization, vaccine antigens are presented to the immune

system simultaneously with highly immunogenic Ad capsid proteins. The major problem with these adenovirus vectors is that the immune responses generated are less likely to be directed to the desired vaccine epitopes (McMichael, *et al.* Nat Rev Immunol 2/283-291 (2002)) and more likely to be directed to the adenovirus-derived antigens, *i.e.*, antigenic competition. There is controversy about the mechanism by which First Generation adenovirus vectors are potent immunogens. It has been hypothesized that the composition of the Ad capsid or a toxic effect of viral genes creates generalized inflammation resulting in a nonspecific immune stimulatory effect. The E1 proteins of Ad act to inhibit inflammation following infection (Schaack, *et al.* PNAS 101/3124-3129 (2004)). Removal of the gene segments for these proteins, which is the case for First Generation adenovirus vectors, results in increased levels of inflammation (Schaack, *et al.* PNAS 101/3124-3129 (2004); Schaack, *et al.* Viral Immunol 18/79-88 (2005)). It has been reported that adenovirus vectors efficiently infect antigen-presenting cells (APC) such as dendritic cells and less immunogenic viral vectors do not (Jooss, *et al.* J Virol 72/4212-4223 (1998)). Antigen presenting cells (APC), such as dendritic cells, are responsible for initiation of CMI responses (Kirk, *et al.* Hum Gene Ther 11/797-806 (2000)). It has been reported that prevention of gene expression in dendritic cells greatly reduces the intensity of the CMI response (Hartigan-O'Connor, *et al.* Mol Ther 4/525-533 (2001)).

Thus, it is apparent from these facts that there remains a need for a more effective vaccine vector candidate. In particular, there remains a need in the art for Ad vaccine vectors that allow multiple vaccinations and vaccinations in individuals with preexisting immunity to Ad. In addition, there is no homologous vaccine delivery vector that can be employed in a prime reimmunization protocol for vaccination. The present invention provides this and other advantages.

BRIEF SUMMARY

One aspect of the invention provides a method of generating an immune response against one or more target antigens in an individual comprising administering to the individual an adenovirus vector comprising: a) a replication

defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding the one or more target antigens; and readministering the adenovirus vector at least once to the individual; thereby generating an immune response against the one or more target antigens.

Another aspect of the invention provides a method for generating an immune response against one or more target antigens in an individual, wherein the individual has preexisting immunity to adenovirus, comprising: administering to the individual an adenovirus vector comprising: a) a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding the one or more target antigens; and readministering the adenovirus vector at least once to the individual; thereby generating an immune response against the one or more target antigens.

In one embodiment of the methods described herein, the target antigen comprises an HIV protein, herpes simplex virus protein, hepatitis C virus protein, malaria protein, plague protein, *M. tuberculosis protein,* or a *Streptococcus pneumonia protein,* or an immunogenic fragment thereof. In certain embodiments, the HIV protein is an HIV-gag protein. In a further embodiment, the target antigen comprises and antigen derived from a Venezuelan Equine Encephalitis Virus (VEEV), Western Equine Encephalitis Virus, or Japanese Encephalitis Virus protein. In yet further embodiments, the target antigen comprises a Leishmania protein, a cancer protein such as carcinoembryonic antigen, Her2Nu, or WT-1.

In one embodiment, the target antigen comprises an antigen derived from an influenza virus protein. In this regard, the influenza protein may be derived from the H5N1 influenza virus. In a further embodiment, the influenza virus protein may be derived from any influenza virus, including but not limited to H3N2, H9N1, H1N1, H2N2, H7N7, H1N2, H9N2, H7N2, H7N3, or H10N7. In certain embodiments, the influenza virus protein may be any influenza protein, including but not limited to, haemagglutinin, neuraminidase, or matrix protein M1.

A further aspect of the invention provides a method of generating an immune response against one or more target antigens in an individual comprising:

administering to the individual a first adenovirus vector comprising: a) a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding at least one target antigen; administering to the individual a second adenovirus vector comprising: a) a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding at least one target antigen, wherein the at least one target antigen of the second adenovirus vector is the same or different from the at least one target antigen of the first adenovirus vector; thereby generating an immune response against one or more target antigens.

In one embodiment of the methods provided herein, the adenovirus vector is not a gutted vector. In another embodiment of the methods provided herein, the individual has preexisting immunity to adenovirus. In a further embodiment, the at least one target antigen of the first and the second adenovirus vectors are derived from the same infectious organism. In another embodiment, the at least one target antigen of the first and the second adenovirus vectors are derived from different infectious organisms.

In yet another embodiment of the methods of the invention, the at least one target antigen of the first adenovirus vector comprises an HIV protein. In a further embodiment, the at least one target antigen of the first adenovirus vector comprises an HIV protein and the at least one target antigen of the second adenovirus vector comprises an HIV protein. In another embodiment, the at least one target antigen of the first adenovirus vector comprises an HIV protein and the at least one target antigen of the second adenovirus vector comprises an HIV protein that is different from the HIV protein of the first adenovirus vector. In a further embodiment, the HIV protein of the first or second adenovirus vector is an HIV-gag protein. In a yet further embodiment, the first adenovirus vector comprises an HIV-gag protein and the second adenovirus vector comprises β-galactosidase. In certain embodiments, the β-galactosidase is an *E. coli* β-galactosidase.

In certain embodiments of the methods provided herein, the at least one target antigen of the first adenovirus vector comprises a cancer protein (*e.g.*, a

Her2/neu antigen) or a carcinoembryonic protein. In other embodiments, the at least one target antigen of the first adenovirus vector comprises a bacterial antigen, a viral antigen, an antigen derived from a protozoan protein, an antigen derived from a fungal protein, an antigen derived from a mold protein, an antigen derived from any mammalian protein, or an antigen derived from an avian protein.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 is a bar graph showing antibody levels from mice immunized with Ad5Null. Mice were immunized three times with Ad5Null viral particles at 14 day intervals. Note the presence of increasing anti-Ad antibody levels after each immunization.

Figure 2 is a bar graph showing neutralizing antibody levels from mice immunized with Ad5Null. Mice were immunized three times with Ad5Null viral particles at 14 day intervals. Note the presence of increasing neutralizing antibodies after each immunization. Optical density readings indicate the presence of viable target cells.

Figure 3 shows the measured levels of antibody in immunized mice. Mice were injected three times with E2b deleted adenovirus vector containing the HIV-gag gene. Note the presence of significantly ($P<0.05$) elevated levels of Gag IgG antibody in experimental mice as compared to normal control mice. Horizontal bars represent the mean value.

Figure 4 is a graph showing antibody levels over time in mice immunized with two different E2b deleted adenovirus vectors each with a different target antigen. Mice were immunized with E2b deleted adenovirus vector containing the HIV-gag gene three times at 14 day intervals. Four weeks later, the same group of mice was immunized two additional times at 14 day intervals with E2b deleted adenovirus vector containing the *Escherichia coli* β-galactosidase gene. Note the presence of increasing levels of HIV-Gag IgG antibody levels after multiple immunizations as compared to pre-immunization levels. Moreover, note the

presence of increasing levels of β-galactosidase IgG antibody after two injections in the same group of mice as compared to pre-immunization levels.

Figure 5A and Figure 5B are bar graphs showing numbers of T-cells expressing IFN-γ and IL-2, respectively. Mice were immunized with E2b deleted adenovirus vector containing the HIV-gag gene three times at 14 day intervals. Four weeks later, the same group of mice was immunized two additional times at 14 day intervals with E2b deleted adenovirus vector containing the β-galactosidase gene. To assess cell-mediated immune responses, ELISPOT assays were performed to determine the number of interferon-γ (IFN-γ) or interleukin-2 (IL-2) secreting cells following stimulation with the HIV-Gag protein, β-galactosidase, or Ad5Null virus. The data are expressed as the number of spot forming cells (SFC) per 10^6 splenocytes. Note the number of IFN-γ and IL-2 producing cells after splenocytes were stimulated.

Figure 6 is a bar graph showing the Ad5 neutralizing antibody (NAb) titers in non-human primates (NHP) during the vaccination protocol using Ad5 [E1-, E2b-]-gag. Three NHP were injected with a single does of 10^{10} VP viable wild type Ad5. Ad5 NAb was measured 30 days after administration and the NHP titers were ≥1:50. The Ad5 immune NHP were then immunized three times on days 0, 27, and 58 with Ad5 [E1-, E2b-]-gag (10^{10} VP/dose). Note the increasing levels of NAb induced during vaccination with Ad5 [E1-, E2b-]-gag. Vertical bars indicate the Standard Error of the Mean (SEM).

Figure 7A and Figure 7B are bar graphs showing numbers of peripheral blood mononuclear cells (PBMCs) in Ad5 immune cynomolgus macaques secreting IFN-γ and IL-2, respectively. PBMCs from individual NHP were collected and cell-mediated immune (CMI) responses were assayed 32 days (Day 90) after the final immunization with Ad5 [E1-, E2b-]-gag. Note the significantly ($P<0.05$) elevated levels of IFN-γ (Figure 7A) and IL-2 (Figure 7B) secreting cells from the PBMC sample taken after the vaccination protocol as compared to a baseline sample (Day –8) taken before vaccinations. The ELISpot data are expressed as the number of spot forming cells (SFC) per 10^6 PBMCs. Vertical bars indicate the SEM.

DETAILED DESCRIPTION

The present invention relates to methods and adenovirus vectors for generating immune responses against target antigens. In particular, the present invention provides an improved Ad-based vaccine such that multiple vaccinations against more than one antigenic target entity can be achieved. Importantly, vaccination can be performed in the presence of preexisting immunity to the Ad and/or administered to subjects previously immunized multiple times with the adenovirus vector of the present invention or other adenovirus vectors. The adenovirus vectors of the invention can be administered to subjects multiple times to induce an immune response against an antigen of interest, including but not limited to, the production of antibodies and cell-mediated immune responses against one or more target antigens.

To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

The term “adenovirus” or “Ad” refers to a group of non-enveloped DNA viruses from the family *Adenoviridae.* In addition to human hosts, these viruses can be found in, but are not limited to, avian, bovine, porcine and canine species. The present invention contemplates the use of any adenovirus from any of the four genera of the family *Adenoviridae* (*e.g., Aviadenovirus, Mastadenovirus, Atadenovirus* and *Siadenovirus*) as the basis of an E2b deleted virus vector, or vector containing other deletions as described herein. In addition, several serotypes are found in each species. Ad also pertains to genetic derivatives of any of these viral serotypes, including but not limited to, genetic mutation, deletion or transposition of homologous or heterologous DNA sequences.

A "helper adenovirus" or "helper virus" refers to an Ad that can supply viral functions that a particular host cell cannot (the host may provide Ad gene products such as E1 proteins). This virus is used to supply, in trans, functions (*e.g.*, proteins) that are lacking in a second virus, or helper dependent virus (*e.g.*, a gutted or gutless virus, or a virus deleted for a particular region such as E2b or other region as desribed herein); the first replication-incompetent virus is said to "help" the

second, helper dependent virus thereby permitting the production of the second viral genome in a cell.

The term “Adenovirus5 null (Ad5null)”, as used herein, refers to a non-replicating Ad that does not contain any heterologous nucleic acid sequences for expression.

The term “First Generation adenovirus”, as used herein, refers to an Ad that has the early region 1 (E1) deleted. In additional cases, the nonessential early region 3 (E3) may also be deleted.

The term “gutted” or “gutless”, as used herein, refers to an adenovirus vector that has been deleted of all viral coding regions.

The term "transfection" as used herein refers to the introduction of foreign nucleic acid into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign nucleic acid, DNA or RNA, into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign DNA into the genomic DNA.

The term "reporter gene" indicates a nucleotide sequence that encodes a reporter molecule (including an enzyme). A "reporter molecule" is detectable in any of a variety of detection systems, including, but not limited to enzyme-based detection assays (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. In one embodiment, the present invention contemplates the *E. coli* β-galactosidase gene (available from Pharmacia Biotech, Pistacataway, N.J.), green fluorescent protein (GFP) (commercially available from Clontech, Palo Alto, Calif.), the human placental alkaline phosphatase gene, the chloramphenicol acetyltransferase (CAT) gene; other reporter genes are known to the art and may be employed.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The nucleic acid sequence thus codes for the amino acid sequence.

The term "heterologous nucleic acid sequence", as used herein, refers to a nucleotide sequence that is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous nucleic acid may include a nucleotide sequence that is naturally found in the cell into which it is introduced or the heterologous nucleic acid may contain some modification relative to the naturally occurring sequence.

The term "transgene" refers to any gene coding region, either natural or heterologous nucleic acid sequences or fused homologous or heterologous nucleic acid sequences, introduced into the cells or genome of a test subject. In the current invention, transgenes are carried on any viral vector that is used to introduce the transgenes to the cells of the subject.

The term "Second Generation Adenovirus", as used herein, refers to an Ad that has all or parts of the E1, E2, E3, and, in certain embodiments, E4 DNA gene sequences deleted (removed) from the virus.

The term "subject", as used herein, refers to any animal, *e.g.*, a mammal or marsupial. Subjects of the present invention include but are not limited to humans, non-human primates (*e.g.,* rhesus or other types of macaques), mice, pigs, horses, donkeys, cows, sheep, rats and fowl of any kind.

Adenovirus Vectors

Compared to First Generation adenovirus vectors, the Second Generation E2b deleted adenovirus vectors of the present invention contain additional deletions in the DNA polymerase gene (pol) and deletions of the pre-terminal protein (pTP). E2b deleted vectors have up to a 13 kb gene-carrying

capacity as compared to the 5 to 6 kb capacity of First Generation adenovirus vectors, easily providing space for nucleic acid sequences encoding any of a variety of target antigens, including for example, the Gag, Pol and Nef genes of HIV (Amalfitano, et al. Curr Gene Ther 2/111-133 (2002)). The E2b deleted adenovirus vectors also have reduced adverse reactions as compared to First Generation adenovirus vectors (Morral, *et al* Hum Gene Ther 9/2709-2716 (1998); Hodges, *et al.* J Gene Med 2/250-259 (2000); DelloRusso, *et al.* Proc Natl Acad Sci USA 99/12979-12984 (2002); Reddy, *et al.* Mol Ther 5/63-73 (2002); (Amalfitano and Parks, *et al.* Curr Gene Ther 2/111-133 (2002); Amalfitano Curr Opin Mol Ther 5/362-366 (2003); Everett, *et al.* Human Gene Ther 14/1715-1726 (2003)) E2b deleted vectors have reduced expression of viral genes (Hodges, *et al.* J Gene Med 2/250-259 (2000); Amalfitano, *et al.* J Virol 72/926-933 (1998); Hartigan-O'Connor, *et al.* Mol Ther 4/525-533 (2001)), and this characteristic has been reported to lead to extended transgene expression *in vivo* (Hu, *et al.* Hum Gene Ther 10/355-364 (1999); DelloRusso, *et al.* Proc Natl Acad Sci USA 99/12979-12984 (2002); Reddy, *et al.* Mol Ther 5/63-73 (2002); (Amalfitano and Parks, *et al.* Curr Gene Ther 2/111-133 (2002); Amalfitano Curr Opin Mol Ther 5/362-366 (2003); Everett, *et al.* Human Gene Ther 14/1715-1726 (2003)).

The innate immune response to wild type Ad can be complex and it appears that Ad proteins expressed from adenovirus vectors play an important role (Moorhead, *et al.* J Virol 73/1046-1053 (1999); Nazir, *et al.* J Investig Med 53/292-304 (2005); Schaack, *et al.* Proc Natl Acad Sci USA 101/3124-3129 (2004); Schaack, *et al.* Viral Immunol 18/79-88 (2005); Kiang, *et al.* Mol Ther 14/588-598 (2006); Hartman, *et al.* J Virol 81/1796-1812 (2007); Hartman, *et al.* Virology 358/357-372 (2007)). Specifically, the deletions of pre-terminal protein and DNA polymerase in the E2b deleted vectors appear to reduce inflammation during the first 24 to 72 hours following injection, whereas First Generation adenovirus vectors stimulate inflammation during this period (Schaack, *et al.* Proc Natl Acad Sci USA 101/3124-3129 (2004); Schaack, *et al.* Viral Immunol 18/79-88 (2005); Kiang, *et al.* Mol Ther 14/588-598 (2006); Hartman, *et al.* J Virol 81/1796-1812 (2007); Hartman,

et al. Virology 358/357-372 (2007)). In addition, it has been reported that the additional replication block created by E2b deletion also leads to a 10,000 fold reduction in expression of Ad late genes, well beyond that afforded by E1, E3 deletions alone (Amalfitano *et al.* J. Virol. 72/926-933 (1998); Hodges *et al.* J. Gene Med. 2/250-259 (2000)). The decreased levels of Ad proteins produced by E2b deleted adenovirus vectors effectively reduce the potential for competitive, undesired, immune responses to Ad antigens, responses that prevent repeated use of the platform in Ad immunized or exposed individuals. The reduced induction of inflammatory response by Second Generation E2b deleted vectors results in increased potential for the vectors to express desired vaccine antigens during the infection of antigen presenting cells (*i.e.* dendritic cells), decreasing the potential for antigenic competition, resulting in greater immunization of the vaccine to the desired antigen relative to identical attempts with First Generation adenovirus vectors. E2b deleted adenovirus vectors provide an improved Ad-based vaccine candidate that is safer, more effective, and more versatile than previously described vaccine candidates using First Generation adenovirus vectors.

Thus, the present invention contemplates the use of E2b deleted adenovirus vectors, such as those described in US Patent Nos. 6,063,622; 6,451,596; 6,057,158: and 6,083,750. As described in the '622 patent, in order to further cripple viral protein expression, and also to decrease the frequency of generating replication competent Ad (RCA), the present invention provides adenovirus vectors containing deletions in the E2b region. Propagation of these E2b deleted adenovirus vectors requires cell lines that express the deleted E2b gene products. The present invention also provides such packaging cell lines; for example E.C7 (formally called C-7), derived from the HEK-203 cell line (Amalfitano, *et al.* Proc Natl Acad Sci USA 93/3352-3356 (1996); Amalfitano, *et al.* Gene Ther 4/258-263 (1997)).

Further, the E2b gene products, DNA polymerase and preterminal protein, can be constitutively expressed in E.C7, or similar cells along with the E1 gene products. Transfer of gene segments from the Ad genome to the production

cell line has immediate benefits: (1) increased carrying capacity of the recombinant DNA polymerase and preterminal protein-deleted adenovirus vector, since the combined coding sequences of the DNA polymerase and preterminal proteins that can be theoretically deleted approaches 4.6 kb; and, (2) a decreased potential of RCA generation, since two or more independent recombination events would be required to generate RCA. Therefore, the E1, Ad DNA polymerase and preterminal protein expressing cell lines used in the present invention enable the propagation of adenovirus vectors with a carrying capacity approaching 13 kb, without the need for a contaminating helper virus [Mitani *et al.* (1995) Proc. Natl. Acad. Sci. USA 92:3854; Hodges, *et al.*, 2000 J Gene Med 2:250-259; (Amalfitano and Parks, Curr Gene Ther 2/111-133 (2002)]. In addition, when genes critical to the viral life cycle are deleted (*e.g.*, the E2b genes), a further crippling of Ad to replicate or express other viral gene proteins occurs. This will decrease immune recognition of virally infected cells, and allows for extended durations of foreign transgene expression.

The most important attribute of E1, DNA polymerase, and preterminal protein deleted vectors, however, is their inability to express the respective proteins from the E1 and E2b regions, as well as a predicted lack of expression of most of the viral structural proteins. For example, the major late promoter (MLP) of Ad is responsible for transcription of the late structural proteins L1 through L5 [Doerfler, In Adenovirus DNA, The Viral Genome and Its Expression (Martinus Nijhoff Publishing Boston, 1986)]. Though the MLP is minimally active prior to Ad genome replication, the highly toxic Ad late genes are primarily transcribed and translated from the MLP only after viral genome replication has occurred [Thomas and Mathews (1980) Cell 22:523]. This cis-dependent activation of late gene transcription is a feature of DNA viruses in general, such as in the growth of polyoma and SV-40. The DNA polymerase and preterminal proteins are absolutely required for Ad replication (unlike the E4 or protein IX proteins) and thus their deletion is extremely detrimental to adenovirus vector late gene expression, and the toxic effects of that expression in cells such as APCs.

In certain embodiments, the adenovirus vectors contemplated for use in the present invention include E2b deleted adenovirus vectors that have a deletion in the E2b region of the Ad genome and the E1 region but do not have any other regions of the Ad genome deleted. In another embodiment, the adenovirus vectors contemplated for use in the present invention include E2b deleted adenovirus vectors that have a deletion in the E2b region of the Ad genome and deletions in the E1 and E3 regions, but no other regions deleted. In a further embodiment, the adenovirus vectors contemplated for use in the present invention include adenovirus vectors that have a deletion in the E2b region of the Ad genome and deletions in the E1, E3 and partial or complete removal of the E4 regions but no other deletions. In another embodiment, the adenovirus vectors contemplated for use in the present invention include adenovirus vectors that have a deletion in the E2b region of the Ad genome and deletions in the E1 and E4 regions but no other deletions. In an additional embodiment, the adenovirus vectors contemplated for use in the present invention include adenovirus vectors that have a deletion in the E2a, E2b and E4 regions of the Ad genome but no other deletions. In one embodiment, the adenovirus vectors for use herein comprise vectors having the E1 and DNA polymerase functions of the E2b region deleted but no other deletions. In a further embodiment, the adenovirus vectors for use herein have the E1 and the preterminal protein functions of the E2b region deleted and no other deletions. In another embodiment, the adenovirus vectors for use herein have the E1, DNA polymerase and the preterminal protein functions deleted, and no other deletions. In one particular embodiment, the adenovirus vectors contemplated for use herein are deleted for at least a portion of the E2b region and the E1 region, but are not "gutted" adenovirus vectors. In this regard, the vectors may be deleted for both the DNA polymerase and the preterminal protein functions of the E2b region. In an additional embodiment, the adenovirus vectors for use in the present invention include adenovirus vectors that have a deletion in the E1, E2b and 100K regions of the adenovirus genome. In one embodiment, the adenovirus vectors for use herein comprise vectors having the E1, E2b and protease functions deleted but no other

deletions. In a further embodiment, the adenovirus vectors for use herein have the E1 and the E2b regions deleted, while the fiber genes have been modified by mutation or other alterations (for example to alter Ad tropism). Removal of genes from the E3 or E4 regions may be added to any of the mentioned adenovirus vectors. In certain embodiments, the adenovirus vector may be a "gutted" adenovirus vector.

The term "E2b deleted", as used herein, refers to a specific DNA sequence that is mutated in such a way so as to prevent expression and/or function of at least one E2b gene product. Thus, in certain embodiments, "E2b deleted" refers to a specific DNA sequence that is deleted (removed) from the Ad genome. E2b deleted or "containing a deletion within the E2b region" refers to a deletion of at least one base pair within the E2b region of the Ad genome. Thus, in certain embodiments, more than one base pair is deleted and in further embodiments, at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 base pairs are deleted. In another embodiment, the deletion is of more than 150, 160, 170, 180, 190, 200, 250, or 300 base pairs within the E2b region of the Ad genome. An E2b deletion may be a deletion that prevents expression and/or function of at least one E2b gene product and therefore, encompasses deletions within exons encoding portions of E2b-specific proteins as well as deletions within promoter and leader sequences. In certain embodiments, an E2b deletion is a deletion that prevents expression and/or function of one or both of the DNA polymerase and the preterminal protein of the E2b region. In a further embodiment, "E2b deleted" refers to one or more point mutations in the DNA sequence of this region of an Ad genome such that one or more encoded proteins is non-functional. Such mutations include residues that are replaced with a different residue leading to a change in the amino acid sequence that result in a nonfunctional protein.

As would be understood by the skilled artisan upon reading the present disclosure, other regions of the Ad genome can be deleted. Thus to be "deleted" in a particular region of the Ad genome, as used herein, refers to a specific DNA sequence that is mutated in such a way so as to prevent expression and/or function

of at least one gene product encoded by that region. In certain embodiments, to be "deleted" in a particular region refers to a specific DNA sequence that is deleted (removed) from the Ad genome in such a way so as to prevent the expression and/or the function encoded by that region (*e.g.*, E2b functions of DNA polymerase or preterminal protein function). "Deleted" or "containing a deletion" within a particular region refers to a deletion of at least one base pair within that region of the Ad genome. Thus, in certain embodiments, more than one base pair is deleted and in further embodiments, at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 base pairs are deleted from a particular region. In another embodiment, the deletion is more than 150, 160, 170, 180, 190, 200, 250, or 300 base pairs within a particular region of the Ad genome. These deletions are such that expression and/or function of the gene product encoded by the region is prevented. Thus deletions encompass deletions within exons encoding portions of proteins as well as deletions within promoter and leader sequences. In a further embodiment, "deleted" in a particular region of the Ad genome refers to one or more point mutations in the DNA sequence of this region of an Ad genome such that one or more encoded proteins is non-functional. Such mutations include residues that are replaced with a different residue leading to a change in the amino acid sequence that result in a nonfunctional protein.

The deleted adenovirus vectors of the present invention can be generated using recombinant techniques known in the art (see *e.g.*, Amalfitano *et al.*, 1998 J. Virol. 72:926-933; Hodges, *et al.*, 2000 J Gene Med 2:250-259).

As would be recognized by the skilled artisan, the adenovirus vectors for use in the present invention can be successfully grown to high titers using an appropriate packaging cell line that constitutively expresses E2b gene products and products of any of the necessary genes that may have been deleted. In certain embodiments, HEK-293-derived cells that not only constitutively express the E1 and DNA polymerase proteins, but also the Ad-preterminal protein, can be used. In one embodiment, E.C7 cells are used to successfully grow high titer stocks of the

adenovirus vectors (see *e.g.*, Amalfitano *et al.*, J. Virol. 1998 72:926-933; Hodges, *et al.* J Gene Med 2/250-259 (2000))

In order to delete critical genes from self-propagating adenovirus vectors, the proteins encoded by the targeted genes have to first be coexpressed in HEK-293 cells, or similar, along with the E1 proteins. Therefore, only those proteins which are non-toxic when coexpressed constitutively (or toxic proteins inducibly-expressed) can be utilized. Coexpression in HEK-293 cells of the E1 and E4 genes has been demonstrated (utilizing inducible, not constitutive, promoters) [Yeh *et al.* (1996) J. Virol. 70:559; Wang *et al.* (1995) Gene Therapy 2:775; and Gorziglia *et al.* (1996) J. Virol. 70:4173]. The E1 and protein IX genes (a virion structural protein) have been coexpressed [Caravokyri and Leppard (1995) J. Virol. 69:6627], and coexpression of the E1, E4, and protein IX genes has also been described [Krougliak and Graham (1995) Hum. Gene Ther. 6:1575]. The E1 and 100k genes have been successfully expressed in transcomplementing cell lines, as have E1 and protease genes (Oualikene, *et al.* Hum Gene Ther 11/1341-1353 (2000); Hodges, *et al.* J. Virol 75/5913-5920 (2001)).

Cell lines coexpressing E1 and E2b gene products for use in growing high titers of E2b deleted Ad particles are described in US Patent No. 6,063,622. The E2b region encodes the viral replication proteins which are absolutely required for Ad genome replication [Doerfler, supra and Pronk *et al.* (1992) Chromosoma 102:S39-S45]. Useful cell lines constitutively express the approximately 140 kD Ad-DNA polymerase and/or the approximately 90 kD preterminal protein. In particular, cell lines that have high-level, constitutive coexpression of the E1, DNA polymerase, and preterminal proteins, without toxicity (*e.g.* E.C7), are desirable for use in propagating Ad for use in multiple vaccinations. These cell lines permit the propagation of adenovirus vectors deleted for the E1, DNA polymerase, and preterminal proteins.

The recombinant Ad of the present invention can be propagated using techniques known in the art. For example, in certain embodiments, tissue culture plates containing E.C7 cells are infected with the adenovirus vector virus stocks at

an appropriate MOI (*e.g.*, 5) and incubated at 37.0°C for 40-96 h. The infected cells are harvested, resuspended in 10 mM Tris-Cl (pH 8.0), and sonicated, and the virus is purified by two rounds of cesium chloride density centrifugation. In certain techniques, the virus containing band is desalted over a Sephadex CL-6B column (Pharmacia Biotech, Piscataway, N.J.), sucrose or glycerol is added, and aliquots are stored at -80°C. In some embodiments, the virus will be placed in a solution designed to enhance its stability, such as A195 (Evans, *et al.* J Pharm Sci 93/2458-2475 (2004))The titer of the stock is measured (*e.g.*, by measurement of the optical density at 260 nm of an aliquot of the virus after SDS lysis). In another embodiment, plasmid DNA, either linear or circular, encompassing the entire recombinant E2b deleted adenovirus vector can be transfected into E.C7, or similar cells, and incubated at 37.0°C until evidence of viral production is present (*e.g.* the cytopathic effect). The conditioned media from these cells can then be used to infect more E.C7, or similar cells, to expand the amount of virus produced, before purification. Purification can be accomplished by two rounds of cesium chloride density centrifugation or selective filtration. In certain embodiments, the virus may be purified by column chromatography, using commercially available products (*e.g.* Adenopure from Puresyn, Inc., Malvern, PA) or custom made chromatographic columns.

Generally, the recombinant Ad of the present invention comprises enough of the virus to ensure that the cells to be infected are confronted with a certain number of viruses. Thus, the present invention provides a stock of recombinant Ad, preferably an RCA-free stock of recombinant Ad. The preparation and analysis of Ad stocks is well known in the art. Viral stocks vary considerably in titer, depending largely on viral genotype and the protocol and cell lines used to prepare them. The viral stocks of the present invention can have a titer of at least about 10^6, 10^7, or 10^8 pfu/ml, and many such stocks can have higher titers, such as at least about 10^9, 10^{10}, 10^{11}, or 10^{12} pfu/ml. Depending on the nature of the recombinant virus and the packaging cell line, it is possible that a viral stock of the present invention can have a titer of even about 10^{13} particles/ml or higher.

Further information on viral delivery systems is known in the art and can be found, for example, in Fisher-Hoch *et al.*, *Proc. Natl. Acad. Sci. USA 86*:317-321, 1989; Flexner *et al.*, *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner *et al.*, *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld *et al.*, *Science 252*:431-434, 1991; Kolls *et al.*, *Proc. Natl. Acad. Sci. USA 91*:215-219, 1994; Kass-Eisler *et al.*, *Proc. Natl. Acad. Sci. USA 90*:11498-11502, 1993; Guzman *et al.*, *Circulation 88*:2838-2848, 1993; and Guzman *et al.*, *Cir. Res. 73*:1202-1207, 1993.

Heterologous Nucleic Acid

The adenovirus vectors of the present invention also comprise heterologous nucleic acid sequences that encode one or more target antigens of interest, fragments or fusions thereof, against which it is desired to generate an immune response. In some embodiments, the adenovirus vectors of the present invention comprise heterologous nucleic acid sequences that encode one or more proteins, fusions thereof or fragments thereof, that can modulate the immune response. In a further embodiment of the invention, the adenovirus vector of the present invention encodes one or more antibodies against specific antigens, such as anthrax protective antigen, permitting passive immunotherapy. In certain embodiments, the adenovirus vectors of the present invention comprise heterologous nucleic acid sequences encoding one or more proteins having therapeutic effect (*e.g.*, anti-viral, anti-bacterial, anti-parasitic, or anti-tumor function). Thus the present invention provides the Second Generation E2b deleted adenovirus vectors that comprise a heterologous nucleic acid sequence.

As such, the present invention further provides nucleic acid sequences, also referred to herein as polynucleotides, that encode one or more target antigens of interest. As such, the present invention provides polynucleotides that encode target antigens from any source as described further herein, vectors comprising such polynucleotides and host cells transformed or transfected with such expression

vectors. The terms "nucleic acid" and "polynucleotide" are used essentially interchangeably herein. As will be also recognized by the skilled artisan, polynucleotides of the invention may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules may include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials. An isolated polynucleotide, as used herein, means that a polynucleotide is substantially away from other coding sequences. For example, an isolated DNA molecule as used herein does not contain large portions of unrelated coding DNA, such as large chromosomal fragments or other functional genes or polypeptide coding regions. Of course, this refers to the DNA molecule as originally isolated, and does not exclude genes or coding regions later added to the segment recombinantly in the laboratory.

As will be understood by those skilled in the art, the polynucleotides of this invention can include genomic sequences, extra-genomic and plasmid-encoded sequences and smaller engineered gene segments that express, or may be adapted to express target antigens as described herein, fragments of antigens, peptides and the like. Such segments may be naturally isolated, or modified synthetically by the hand of man.

Polynucleotides may comprise a native sequence (*i.e.,* an endogenous sequence that encodes a target antigen polypeptide/protein/epitope of the invention or a portion thereof) or may comprise a sequence that encodes a variant or derivative of such a sequence. In certain embodiments, the polynucleotide sequences set forth herein encode target antigen proteins as described herein. In some embodiments, polynucleotides represent a novel gene sequence that has been optimized for expression in specific cell types (*i.e.* human cell lines) that may

substantially vary from the native nucleotide sequence or variant but encode a similar protein antigen.

In other related embodiments, the present invention provides polynucleotide variants having substantial identity to native sequences encoding proteins (*e.g.*, target antigens of interest) as described herein, for example those comprising at least 70% sequence identity, preferably at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or higher, sequence identity compared to a native polynucleotide sequence encoding the polypeptides of this invention using the methods described herein, (*e.g.*, BLAST analysis using standard parameters, as described below). One skilled in this art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like.

Typically, polynucleotide variants will contain one or more substitutions, additions, deletions and/or insertions, preferably such that the immunogenicity of the epitope of the polypeptide encoded by the variant polynucleotide or such that the immunogenicity of the heterologous target protein is not substantially diminished relative to a polypeptide encoded by the native polynucleotide sequence. As described elsewhere herein, the polynucleotide variants preferably encode a variant of the target antigen, or a fragment (*e.g.*, an epitope) thereof wherein the propensity of the variant polypeptide or fragment (*e.g.*, epitope) thereof to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished relative to the native polypeptide. The term "variants" should also be understood to encompass homologous genes of xenogenic origin.

The present invention provides polynucleotides that comprise or consist of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 11, 120, 130, 140, 150,160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 or more contiguous nucleotides encoding a polypeptide, including target protein antigens, as described herein, as well as all intermediate lengths there

between. It will be readily understood that "intermediate lengths", in this context, means any length between the quoted values, such as 16, 17, 18, 19, *etc.*; 21, 22, 23, *etc.*; 30, 31, 32, *etc.*; 50, 51, 52, 53, *etc.*; 100, 101, 102, 103, *etc.*; 150, 151, 152, 153, *etc.*; including all integers through 200-500; 500-1,000, and the like. A polynucleotide sequence as described herein may be extended at one or both ends by additional nucleotides not found in the native sequence encoding a polypeptide as described herein, such as an epitope or heterologous target protein. This additional sequence may consist of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides or more, at either end of the disclosed sequence or at both ends of the disclosed sequence.

The polynucleotides of the present invention, or fragments thereof, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, expression control sequences, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, illustrative polynucleotide segments with total lengths of about 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, about 500, about 200, about 100, about 50 base pairs in length, and the like, (including all intermediate lengths) are contemplated to be useful in many implementations of this invention.

When comparing polynucleotide sequences, two sequences are said to be "identical" if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence

may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J., *Unified Approach to Alignment and Phylogenes*, pp. 626-645 (1990); *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M., *CABIOS 5*:151-153 (1989); Myers, E.W. and Muller W., *CABIOS 4*:11-17 (1988); Robinson, E.D., *Comb. Theor 11*:105 (1971); Saitou, N. Nei, M., *Mol. Biol. Evol. 4*:406-425 (1987); Sneath, P.H.A. and Sokal, R.R., *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA (1973); Wilbur, W.J. and Lipman, D.J., *Proc. Natl. Acad., Sci. USA 80*:726-730 (1983).

Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman, *Add. APL. Math* 2:482 (1981), by the identity alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity methods of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nucl. Acids Res.* 25:3389-3402 (1977), and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine

percent sequence identity for the polynucleotides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. In one illustrative example, cumulative scores can be calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments, (B) of 50, expectation (E) of 10, M=5, N=-4 and a comparison of both strands.

Preferably, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.,* gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (*i.e.,* the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a particular antigen of interest, or fragment thereof, as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon

usage are specifically contemplated by the present invention. Further, alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the present invention. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein may, but need not, have an altered structure or function. Alleles may be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

Therefore, in another embodiment of the invention, a mutagenesis approach, such as site-specific mutagenesis, is employed for the preparation of variants and/or derivatives of the target antigen sequences, or fragments thereof, as described herein. By this approach, specific modifications in a polypeptide sequence can be made through mutagenesis of the underlying polynucleotides that encode them. These techniques provide a straightforward approach to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the polynucleotide.

Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or otherwise change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

In certain embodiments of the present invention, the inventors contemplate the mutagenesis of the disclosed polynucleotide sequences to alter one or more properties of the encoded polypeptide, such as the immunogenicity of an epitope comprised in a polypeptide. Assays to test the immunogenicity of a polypeptide or variant thereof are well known in the art and include, but are not

limited to, T cell cytotoxicity assays (CTL/chromium release assays), T cell proliferation assays, intracellular cytokine staining, ELISA, ELISpot, *etc.* The techniques of site-specific mutagenesis are well known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site-specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25 nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy *et al.*, 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis *et al.*, 1982.

Polynucleotide segments or fragments encoding the polypeptides of the present invention may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U. S. Patent 4,683,202, by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology (see for example, Current Protocols in Molecular Biology, John Wiley and Sons, NY, NY).

In order to express a desired target antigen polypeptide or fragment thereof, or fusion protein comprising any of the above, as described herein, the nucleotide sequences encoding the polypeptide, or functional equivalents, are inserted into an appropriate Ad as described elsewhere herein using recombinant

techniques known in the art. The appropriate adenovirus vector contains the necessary elements for the transcription and translation of the inserted coding sequence and any desired linkers. Methods which are well known to those skilled in the art may be used to construct these adenovirus vectors containing sequences encoding a polypeptide of interest and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Amalfitano *et al.*, 1998 J. Virol. 72:926-933; Hodges, *et al.*, 2000 J Gene Med 2:250-259; Sambrook, J. *et al.* (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., and Ausubel, F. M. *et al.* (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York. N.Y.

A variety of vector/host systems may be utilized to contain and produce polynucleotide sequences. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA vectors; yeast transformed with yeast vectors; insect cell systems infected with virus vectors (*e.g.*, baculovirus); plant cell systems transformed with virus vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial vectors (*e.g.*, Ti or pBR322 plasmids); or animal cell systems.

The "control elements" or "regulatory sequences" present in an adenovirus vector are those non-translated regions of the vector—enhancers, promoters, 5' and 3' untranslated regions—which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, sequences encoding a polypeptide of interest may be ligated into an Ad transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing the polypeptide in infected host cells (Logan, J. and Shenk, T. (1984)

Proc. Natl. Acad. Sci. 81:3655-3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding a polypeptide of interest. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding the polypeptide, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. *et al.* (1994) *Results Probl. Cell Differ. 20*:125-162). Specific termination sequences, either for transcription or translation, may also be incorporated in order to achieve efficient translation of the sequence encoding the polypeptide of choice.

A variety of protocols for detecting and measuring the expression of polynucleotide-encoded products (*e.g.*, target antigens of interest), using either polyclonal or monoclonal antibodies specific for the product are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on a given polypeptide may be preferred for some applications, but a competitive binding assay may also be employed. These and other assays are described, among other places, in Hampton, R. *et al.* (1990; Serological Methods, a Laboratory Manual, APS Press, St Paul. Minn.) and Maddox, D. E. *et al.* (1983; *J. Exp. Med. 158*:1211-1216).

The adenovirus vectors of the present invention comprise nucleic acid sequences encoding one or more antigens of interest. The nucleic acid sequence may also contain a product that can be detected or selected for. As referred to herein, a "reporter" gene is one whose product can be detected, such as by fluorescence, enzyme activity on a chromogenic or fluorescent substrate, and the like or selected for by growth conditions. Such reporter genes include, without limitation, green fluorescent protein (GFP), β-galactosidase, chloramphenicol acetyltransferase (CAT), luciferase, neomycin phosphotransferase, secreted alkaline phosphatase (SEAP), and human growth hormone (HGH). Selectable markers include drug resistances, such as neomycin (G418), hygromycin, and the like.

The nucleic acid encoding an antigen of interest may also comprise a promoter or expression control sequence. This is a nucleic acid sequence that controls expression of the nucleic acid sequence encoding a target antigen and generally is active or activatable in the targeted cell. The choice of the promoter will depend in part upon the targeted cell type and the degree or type of control desired. Promoters that are suitable within the context of the present invention include, without limitation, constitutive, inducible, tissue specific, cell type specific, temporal specific, or event-specific.

Examples of constitutive or nonspecific promoters include the SV40 early promoter (U.S. Patent No. 5,118,627), the SV40 late promoter (U.S. Patent No. 5,118,627), CMV early gene promoter (U.S. Patent No. 5,168,062), bovine papilloma virus promoter, and adenovirus promoter. In addition to viral promoters, cellular promoters are also amenable within the context of this invention. In particular, cellular promoters for the so-called housekeeping genes are useful (*e.g.*, β-actin). Viral promoters are generally stronger promoters than cellular promoters.

Inducible promoters may also be used. These promoters include MMTV LTR (PCT WO 91/13160), inducible by dexamethasone, metallothionein, inducible by heavy metals, and promoters with cAMP response elements, inducible by cAMP, heat shock, promoter. By using an inducible promoter, the nucleic acid

may be delivered to a cell and will remain quiescent until the addition of the inducer. This allows further control on the timing of production of the protein of interest.

Event-type specific promoters are active or upregulated only upon the occurrence of an event, such as tumorigenicity or viral infection, for example. The HIV LTR is a well-known example of an event-specific promoter. The promoter is inactive unless the tat gene product is present, which occurs upon viral infection. Some event-type promoters are also tissue-specific. Preferred event-type specific promoters include promoters activated upon viral infection.

Examples of promoters discussed herein include, but are not limited to, promoters for alphafetoprotein, alpha actin, myo D, carcinoembryonic antigen, VEGF-receptor (GenBank Accession No. X89776); FGF receptor; TEK or tie 2 (GenBank Accession No. L06139); tie (GenBank Accession Nos. X60954; S89716); urokinase receptor (GenBank Accession No. S78532); E- and P-selectins (GenBank Accession Nos. M64485; L01874); VCAM-1 (GenBank Accession No. M92431); endoglin (GenBank Accession No. HSENDOG); endosialin (Rettig *et al.*, PNAS 89:10832, 1992); alpha V-beta3 integrin (Villa-Garcia *et al.*, Blood 3:668, 1994; Donahue *et al.*, BBA 1219:228, 1994); endothelin-1 (GenBank Accession Nos. M25377; J04819; J05489); ICAM-3 (GenBank Accession No. S50015); E9 antigen (Wang *et al.*, Int. J. Cancer 54:363, 1993); von Willebrand factor (GenBank Accession Nos. HUMVWFI;. HUMVWFA); CD44 (GenBank Accession No. HUMCD44B); CD40 (GenBank Accession Nos. HACD40L; HSCD405FR); vascular-endothelial cadherin (Martin-Padura *et al.*, J. Pathol. 175:51, 1995); notch 4 (Uyttendaele *et al.*, Development 122:2251, 1996) high molecular weight melanoma-associated antigen; prostate specific antigen-1, probasin, FGF receptor, VEGF receptor, erb B2; erb B3; erb B4; MUC-1; HSP-27; int-1; int-2, CEA, HBEGF receptor; EGF receptor; tyrosinase, MAGE, IL-2 receptor; prostatic acid phosphatase, probasin, prostate specific membrane antigen, alpha-crystallin, PDGF receptor, integrin receptor, α-actin, SM1 and SM2 myosin heavy chains, calponin-h1, SM22 alpha angiotensin receptor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-

10, IL-11, IL-12, IL-13, IL-14, immunoglobulin heavy chain, immunoglobulin light chain, CD4, and the like are useful within the context of this invention.

In addition to the promoter, repressor sequences, negative regulators, or tissue-specific silencers may be inserted to reduce non-specific expression of the polynucleotide. Multiple repressor elements may be inserted in the promoter region. Repression of transcription is independent of the orientation of repressor elements or distance from the promoter. One type of repressor sequence is an insulator sequence. Such sequences inhibit transcription (Dunaway *et al.*, Mol Cell Biol 17: 182-9, 1997; Gdula *et al.*, Proc Natl Acad Sci USA 93:9378-83, 1996, Chan *et al.*, J Virol 70: 5312-28, 1996; Scott and Geyer, EMBO J 14: 6258-67, 1995; Kalos and Fournier, Mol Cell Biol 15: 198-207, 1995; Chung *et al.*, Cell 74: 505-14, 1993) and will silence background transcription.

Negative regulatory elements have been characterized in the promoter regions of a number of different genes. The repressor element functions as a repressor of transcription in the absence of factors, such as steroids, as does the NSE in the promoter region of the ovalbumin gene (Haecker *et al.*, Mol. Endocrinology 9:1113-1126, 1995). These negative regulatory elements bind specific protein complexes from oviduct, none of which are sensitive to steroids. Three different elements are located in the promoter of the ovalbumin gene. Oligonucleotides corresponding to portions of these elements repress viral transcription of the TK reporter. One of the silencer elements shares sequence identity with silencers in other genes (TCTCTCCNA).

Repressor elements have also been identified in the promoter region of a variety of genes, including the collagen II gene, for example. Gel retardation studies showed that nuclear factors from HeLa cells bind specifically to DNA fragments containing the silencer region, whereas chondrocyte nuclear extracts did not show any binding activity (Savanger *et al.*, J. Biol. Chem. 265(12):6669-6674, 1990). Repressor elements have also been shown to regulate transcription in the carbamyl phosphate synthetase gene (Goping *et al.*, Nucleic Acid Research 23(10):1717-1721, 1995). This gene is expressed in only two different cell types,

hepatocytes and epithelial cells of the intestinal mucosa. Negative regulatory regions have also been identified in the promoter region of the choline acetyltransferase gene, the albumin promoter (Hu *et al.*, J. Cell Growth Differ. 3(9):577-588, 1992), phosphoglycerate kinase (PGK-2) gene promoter (Misuno *et al.*, Gene 119(2):293-297, 1992), and in the 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase gene, in which the negative regulatory element inhibits transcription in non-hepatic cell lines (Lemaigre *et al.*, Mol. Cell Biol. 11(2):1099-1106). Furthermore, the negative regulatory element Tse-1 has been identified in a number of liver specific genes, including tyrosine aminotransferase (TAT). TAT gene expression is liver specific and inducible by both glucocorticoids and the cAMP signaling pathway. The cAMP response element (CRE) has been shown to be the target for repression by Tse-1 and hepatocyte-specific elements (Boshart *et al.*, Cell 61(5):905-916, 1990). Accordingly, it is clear that varieties of such elements are known or are readily identified.

In certain embodiments, elements that increase the expression of the desired target antigen are incorporated into the nucleic acid sequence of the adenovirus vectors described herein. Such elements include internal ribosome binding sites (IRES; Wang and Siddiqui, Curr. Top. Microbiol. Immunol 203:99, 1995; Ehrenfeld and Semler, Curr. Top. Microbiol. Immunol. 203:65, 1995; Rees *et al.*, Biotechniques 20:102, 1996; Sugimoto *et al.*, Biotechnology12:694, 1994). IRES increase translation efficiency. As well, other sequences may enhance expression. For some genes, sequences especially at the 5′ end inhibit transcription and/or translation. These sequences are usually palindromes that can form hairpin structures. Any such sequences in the nucleic acid to be delivered are generally deleted. Expression levels of the transcript or translated product are assayed to confirm or ascertain which sequences affect expression. Transcript levels may be assayed by any known method, including Northern blot hybridization, RNase probe protection and the like. Protein levels may be assayed by any known method, including ELISA.

As would be recognized by the skilled artisan, the adenovirus vectors of the present invention comprising heterologous nucleic acid sequences can be generated using recombinant techniques known in the art, such as those described in Maione *et al.*, 2001 Proc Natl Acad Sci USA, 98:5986-5991; Maione *et al.*, 2000 Hum Gene Ther 11:859-868; Sandig *et al.* 2000 Proc Natl Acad Sci USA, 97:1002-1007; Harui *et al.* 2004 Gene Therapy, 11:1617-1626; Parks *et al.*, 1996 Proc Natl Acad Sci USA, 93:13565-13570; DelloRusso *et al.*, 2002 Proc Natl Acad Sci USA, 99:12979-12984; Current Protocols in Molecular Biology, John Wiley and Sons, NY, NY).

As noted above, the adenovirus vectors of the present invention comprise nucleic acid sequences that encode one or more target proteins or antigens of interest. In this regard, the vectors may contain nucleic acid encoding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more different target antigens of interest. The target antigens may be a full length protein or may be a fragment (*e.g.*, an epitope) thereof. The adenovirus vectors may contain nucleic acid sequences encoding multiple fragments or epitopes from one target protein of interest or may contain one or more fragments or epitopes from numerous different target proteins of interest.

The term "target antigen" or "target protein" as used herein refers to a molecule, such as a protein, against which an immune response is to be directed. The target antigen may comprise any substance against which it is desirable to generate an immune response but generally, the target antigen is a protein. A target antigen may comprise a full length protein or a fragment thereof that induces an immune response (*i.e.*, an immunogenic fragment).

An "immunogenic fragment," as used herein is a fragment of a polypeptide that is specifically recognized (*i.e.,* specifically bound) by a B-cell and/or T-cell surface antigen receptor resulting in the generation of an immune response specifically against the fragment. In certain embodiments, immunogenic fragments bind to an MHC class I or class II molecule. As used herein, an immunogenic fragment is said to "bind to" an MHC class I or class II molecule if such binding is

detectable using any assay known in the art. For example, the ability of a polypeptide to bind to MHC class I may be evaluated indirectly by monitoring the ability to promote incorporation of ^{125}I labeled β2-microglobulin (β2m) into MHC class I/β2m/peptide heterotrimeric complexes (*see* Parker *et al.*, *J. Immunol. 152*:163, 1994). Alternatively, functional peptide competition assays that are known in the art may be employed. Immunogenic fragments of polypeptides may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Representative techniques for identifying immunogenic fragments include screening polypeptides for the ability to react with antigen-specific antisera and/or T-cell lines or clones. An immunogenic fragment of a particular target polypeptide is a fragment that reacts with such antisera and/or T-cells at a level that is not substantially less than the reactivity of the full length target polypeptide (*e.g.,* in an ELISA and/or T-cell reactivity assay). In other words, an immunogenic fragment may react within such assays at a level that is similar to or greater than the reactivity of the full length polypeptide. Such screens may generally be performed using methods well known to those of ordinary skill in the art, such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988.

Target antigens of the present invention include but are not limited to antigens derived from any of a variety of infectious agents or cancer cells. As used herein, an "infectious agent" is any living organism capable of infecting a host and "cancer" means a neoplastic cell. Infectious agents include, for example, bacteria, any variety of viruses, such as, single stranded RNA viruses, single stranded DNA viruses, fungi, parasites, and protozoa. Examples of infectious agents include, but are not limited to, Actinobacillus spp., Actinomyces spp., Adenovirus (types 1, 2, 3, 4, 5 et 7), Adenovirus (types 40 and 41), Aerococcus spp., Aeromonas hydrophila, Ancylostoma duodenale, Angiostrongylus cantonensis, Ascaris lumbricoides, Ascaris spp., Aspergillus spp., Babesia spp, B. microti, Bacillus anthracis, Bacillus cereus, Bacteroides spp., Balantidium coli, Bartonella bacilliformis, Blastomyces dermatitidis,

Bluetongue virus, Bordetella bronchiseptica, Bordetella pertussis, Borrelia afzelii, Borrelia burgdorferi, Borrelia garinii, Branhamella catarrhalis, Brucella spp. (B. abortus, B. canis, B. melitensis, B. suis), Brugia spp., Burkholderia, (Pseudomonas) mallei, Burkholderia (Pseudomonas) pseudomallei, California serogroup, Campylobacter fetus subsp. Fetus, Campylobacter jejuni, C. coli, C. fetus subsp. Jejuni, Candida albicans, Capnocytophaga spp., Chikungunya virus, Chlamydia psittaci, Chlamydia trachomatis, Citrobacter spp., Clonorchis sinensis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Clostridium spp. (with the exception of those species listed above), Coccidioides immitis, Colorado tick fever virus, Corynebacterium diphtheriae, Coxiella burnetii, Coxsackievirus, Creutzfeldt-Jakob agent, Kuru agent, Crimean-Congo hemorrhagic fever virus, Cryptococcus neoformans, Cryptosporidium parvum, Cytomegalovirus, Cyclospora cayatanesis, Dengue virus (1, 2, 3, 4), Diphtheroids, Eastern (Western) equine encephalitis virus, Ebola virus, Echinococcus granulosus, Echinococcus multilocularis, Echovirus, Edwardsiella tarda, Entamoeba histolytica, Enterobacter spp., Enterovirus 70, Epidermophyton floccosum, Ehrlichia spp, Ehrlichia sennetsu, Microsporum spp. Trichophyton spp., Epstein-Barr virus, Escherichia coli, enterohemorrhagic, Escherichia coli, enteroinvasive, Escherichia coli, enteropathogenic, Escherichia coli, enterotoxigenic, Fasciola hepatica, Francisella tularensis, Fusobacterium spp., Gemella haemolysans, Giardia lamblia, Guanarito virus, Haemophilus ducreyi, Haemophilus influenzae (group b), Hantavirus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis D virus, Hepatitis E virus, Herpes simplex virus, Herpesvirus simiae, Histoplasma capsulatum, Human coronavirus, Human immunodeficiency virus, Human papillomavirus, Human rotavirus, Human T-lymphotrophic virus, Influenza virus including H5N1, Junin virus / Machupo virus, Klebsiella spp., Kyasanur Forest disease virus, Lactobacillus spp., Lassa virus, Legionella pneumophila, Leishmania major, Leishmania infantum, Leishmania spp., Leptospira interrogans, Listeria monocytogenes, Lymphocytic choriomeningitis virus, Machupo virus, Marburg virus, Measles virus, Micrococcus spp., Moraxella spp., Mycobacterium spp. (other than M. bovis, M. tuberculosis, M.

avium,, M. leprae), Mycobacterium tuberculosis, M. bovis, Mycoplasma hominis, M. orale, M. salivarium, M. fermentans, Mycoplasma pneumoniae, Naegleria fowleri, Necator americanus, Neisseria gonorrhoeae, Neisseria meningitides, Neisseria spp. (other than N. gonorrhoeae and N. meningitidis), Nocardia spp., Norwalk virus, Omsk hemorrhagic fever virus, Onchocerca volvulus, Opisthorchis spp., Parvovirus B19, Pasteurella spp., Peptococcus spp., Peptostreptococcus spp., Plasmodium falciparum, Plasmodium vivax, Plasmodium spp., Plesiomonas shigelloides, Powassan encephalitis virus, Proteus spp., Pseudomonas spp. (other than P. mallei, P. pseudomallei), Rabies virus, Respiratory syncytial virus, Rhinovirus, Rickettsia akari, Rickettsia prowazekii, R. Canada, Rickettsia rickettsii, Rift Valley virus, Ross river virus / O'Nyong-Nyong virus, Rubella virus, Salmonella choleraesuis, Salmonella paratyphi, Salmonella typhi, Salmonella spp. (with the exception of those species listed above), Schistosoma spp., Scrapie agent, Serratia spp., Shigella spp., Sindbis virus, Sporothrix schenckii, St. Louis encephalitis virus, Murray Valley encephalitis virus, Staphylococcus aureus, Streptobacillus moniliformis, Streptococcus agalactiae, Streptococcus faecalis, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus salivarius, Taenia saginata, Taenia solium, Toxocara canis, T. cati, T. cruzi, Toxoplasma gondii, Treponema pallidum, Trichinella spp., Trichomonas vaginalis, Trichuris trichiura, Trypanosoma brucei, *Trypanosoma cruzi*, Ureaplasma urealyticum, Vaccinia virus, Varicella-zoster virus, eastern equine encephalitis virus (EEEV), severe acute respiratory virus (SARS), Venezuelan equine encephalitis virus (VEEV), Vesicular stomatitis virus, Vibrio cholerae, serovar 01, Vibrio parahaemolyticus, West Nile virus, Wuchereria bancrofti, Yellow fever virus, Yersinia enterocolitica, Yersinia pseudotuberculosis, and Yersinia pestis.

Examples of infectious agents associated with human malignancies include Epstein-Barr virus, Helicobacter pylori, Hepatitis B virus, Hepatitis C virus, Human heresvirus-8, Human immunodeficiency virus, Human papillomavirus, Human T cell leukemia virus, liver flukes, and Schistosoma haematobium.

A number of viruses are associated with viral hemorrhagic fever, including filoviruses (*e.g.*, Ebola, Marburg, and Reston), arenaviruses (*e.g.* Lassa, Junin, and Machupo), and bunyaviruses. In addition, phleboviruses, including, for example, Rift Valley fever virus, have been identified as etiologic agents of viral hemorrhagic fever. Etiological agents of hemorrhagic fever and associated inflammation may also include paramyxoviruses, particularly respiratory syncytial virus (Feldmann, H. *et al.* (1993) *Arch Virol Suppl. 7*:81-100). In addition, other viruses causing hemorrhagic fevers in man have been identified as belonging to the following virus groups: togavirus (Chikungunya), flavivirus (dengue, yellow fever, Kyasanur Forest disease, Omsk hemorrhagic fever), nairovirus (Crimian-Congo hemorrhagic fever) and hantavirus (hemorrhagic fever with renal syndrome, nephropathic epidemia). Furthermore, Sin Nombre virus was identified as the etiologic agent of the 1993 outbreak of hantavirus pulmonary syndrome in the American Southwest.

Target antigens may include proteins produced by any of the infectious organisms described herein, such as, but not limited to, viral coat proteins, *i.e.*, influenza neuraminidase and hemagglutinin, HIV gp160 or derivatives thereof, HIV Gag, HIV Nef, HIV Pol, SARS coat proteins, herpes virion proteins, WNV proteins, *etc.* Target antigens may also include bacterial surface proteins including pneumococcal PsaA, PspA, LytA, surface or virulence associated proteins of bacterial pathogens such as *Nisseria gonnorhea,* outer membrane proteins or surface proteases.

Target antigens of the present invention include but are not limited to antigens derived from a variety of tumor proteins. Illustrative tumor proteins useful in the present invention include, but are not limited to any one or more of, WT1, p53, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, BAGE, DAM-6, -10, GAGE-1, -2, -8, GAGE-3, -4, -5, -6, -7B, NA88-A, NY-ESO-1, MART-1, MC1R, Gp100, PSA, PSM, Tyrosinase, TRP-1, TRP-2, ART-4, CAMEL, CEA, Cyp-B, Her2/neu, BRCA1, hTERT, hTRT, iCE, MUC1, MUC2, PRAME, P15, RU1, RU2, SART-1, SART-3, WT1, AFP, β-catenin/m, Caspase-8/m, CEA, CDK-

4/m, ELF2M, GnT-V, G250, HSP70-2M, HST-2, KIAA0205, MUM-1, MUM-2, MUM-3, Myosin/m, RAGE, SART-2, TRP-2/INT2, 707-AP, Annexin II, CDC27/m, TPI/mbcr-abl, ETV6/AML, LDLR/FUT, Pml/RARα, and TEL/AML1. These and other tumor proteins are known to the skilled artisan.

In certain embodiments tumor antigens may be identified directly from an individual with cancer. In this regard, screens can be carried out using a variety of known technologies. For example, in one embodiment, a tumor biopsy is taken from a patient, RNA is isolated from the tumor cells and screened using a gene chip (for example, from Affymetrix, Santa Clara, CA) and a tumor antigen is identified. Once the tumor target antigen is identified, it may then be cloned, expressed and purified using techniques known in the art. This target molecule is then linked to one or more epitopes/cassettes of the present invention as described herein and administered to the cancer patient in order to alter the immune response to the target molecule isolated from the tumor. In this manner, "personalized vaccines" are contemplated within the context of the invention. In certain embodiments, cancers may include carcinomas or sarcomas.

The adenovirus vectors of the present invention may also include nucleic acid sequences that encode proteins that increase the immunogenicity of the target antigen. In this regard, the protein produced following immunization with the adenovirus vector containing such a protein may be a fusion protein comprising the target antigen of interest fused to a protein that increases the immunogenicity of the target antigen of interest.

In one embodiment, such an "immunological fusion partner" is derived from a Mycobacterium sp., such as a *Mycobacterium tuberculosis*-derived Ra12 fragment. Ra12 compositions and methods for their use in enhancing the expression and/or immunogenicity of heterologous polynucleotide/polypeptide sequences are described in U.S. Patent Application 60/158,585. Briefly, Ra12 refers to a polynucleotide region that is a subsequence of a *Mycobacterium tuberculosis* MTB32A nucleic acid. MTB32A is a serine protease of 32 KD molecular weight encoded by a gene in virulent and avirulent strains of *M. tuberculosis*. The

nucleotide sequence and amino acid sequence of MTB32A have been described (for example, U.S. Patent Application 60/158,585; see also, Skeiky *et al.*, *Infection and Immun. 67*:3998-4007 (1999), incorporated herein by reference). C-terminal fragments of the MTB32A coding sequence express at high levels and remain as a soluble polypeptides throughout the purification process. Moreover, Ra12 may enhance the immunogenicity of heterologous immunogenic polypeptides with which it is fused. One Ra12 fusion polypeptide comprises a 14 KD C-terminal fragment corresponding to amino acid residues 192 to 323 of MTB32A. Other Ra12 polynucleotides generally comprise at least about 15 consecutive nucleotides, at least about 30 nucleotides, at least about 60 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, or at least about 300 nucleotides that encode a portion of a Ra12 polypeptide. Ra12 polynucleotides may comprise a native sequence (*i.e.,* an endogenous sequence that encodes a Ra12 polypeptide or a portion thereof) or may comprise a variant of such a sequence. Ra12 polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the biological activity of the encoded fusion polypeptide is not substantially diminished, relative to a fusion polypeptide comprising a native Ra12 polypeptide. Variants preferably exhibit at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity to a polynucleotide sequence that encodes a native Ra12 polypeptide or a portion thereof.

Within another embodiment, an immunological fusion partner is derived from protein D, a surface protein of the gram-negative bacterium *Haemophilus influenza B* (WO 91/18926). Preferably, a protein D derivative comprises approximately the first third of the protein (*e.g.,* the first N-terminal 100-110 amino acids), and a protein D derivative may be lipidated. Within certain embodiments, the first 109 residues of a Lipoprotein D fusion partner is included on the N-terminus to provide the polypeptide with additional exogenous T-cell epitopes and to increase the expression level in *E. coli* (thus functioning as an expression enhancer). The lipid tail ensures optimal presentation of the antigen to antigen presenting cells.

Other fusion partners include the non-structural protein from influenzae virus, NS1 (hemagglutinin). Typically, the N-terminal 81 amino acids are used, although different fragments that include T-helper epitopes may be used.

In another embodiment, the immunological fusion partner is the protein known as LYTA, or a portion thereof (preferably a C-terminal portion). LYTA is derived from *Streptococcus pneumoniae*, which synthesizes an N-acetyl-L-alanine amidase known as amidase LYTA (encoded by the LytA gene; *Gene 43*:265-292, 1986). LYTA is an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at the amino terminus has been described (*see Biotechnology 10*:795-798, 1992). Within another embodiment, a repeat portion of LYTA may be incorporated into a fusion polypeptide. A repeat portion is found in the C-terminal region starting at residue 178. One particular repeat portion incorporates residues 188-305.

Methods of Use

The adenovirus vectors of the present invention can be used in a number of vaccine settings for generating an immune response against one or more target antigens as described herein. The adenovirus vectors are of particular importance because of the unexpected finding that they can be used to generate immune responses in subjects who have preexisting immunity to Ad and can be used in vaccination regimens that include multiple rounds of immunization using the adenovirus vectors, regimens not possible using previous generation adenovirus vectors.

Generally, generating an immune response comprises an induction of a humoral response and/or a cell-mediated response. In certain embodiments, it is desirable to increase an immune response against a target antigen of interest. In

certain circumstances, generating an immune response may involve a decrease in the activity and/or number of certain cells of the immune system or a decrease in the level and/or activity of certain cytokines or other effector molecules. As such "generating an immune response" or "inducing an immune response" comprises any statistically significant change, *e.g.* increase or decrease, in the number of one or more immune cells (T cells, B cells, antigen-presenting cells, dendritic cells, neutrophils, and the like) or in the activity of one or more of these immune cells (CTL activity, HTL activity, cytokine secretion, change in profile of cytokine secretion, *etc.*).

The skilled artisan would readily appreciate that a number of methods for establishing whether an alteration in the immune response has taken place are available. A variety of methods for detecting alterations in an immune response (*e.g.* cell numbers, cytokine expression, cell activity) are known in the art and are useful in the context of the instant invention. Illustrative methods are described in *Current Protocols in Immunology,* Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober (2001 John Wiley & Sons, NY, NY) Ausubel *et al.* (2001 *Current Protocols in Molecular Biology*, Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., NY, NY); Sambrook *et al.* (1989 *Molecular Cloning*, Second Ed., Cold Spring Harbor Laboratory, Plainview, NY); Maniatis *et al.* (1982 Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, NY) and elsewhere. Illustrative methods useful in this context include intracellular cytokine staining (ICS), ELISpot, proliferation assays, cytotoxic T cell assays including chromium release or equivalent assays, and gene expression analysis using any number of polymerase chain reaction (PCR) or RT-PCR based assays.

In certain embodiments, generating an immune response comprises an increase in target antigen-specific CTL activity of between 1.5 and 5 fold in a subject administered the adenovirus vectors of the invention as compared to a control. In another embodiment, generating an immune response comprises an increase in target-specific CTL activity of about 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 15, 16, 17, 18, 19, 20, or more fold in a subject administered the adenovirus vectors as compared to a control.

In a further embodiment, generating an immune response comprises an increase in target antigen-specific HTL activity, such as proliferation of helper T cells, of between 1.5 and 5 fold in a subject administered the adenovirus vectors of the invention that comprise nucleic acid encoding the target antigen as compared to an appropriate control. In another embodiment, generating an immune response comprises an increase in target-specific HTL activity of about 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 15, 16, 17, 18, 19, 20, or more fold as compared to a control. In this context, HTL activity may comprise an increase as described above, or decrease, in production of a particular cytokine, such as interferon-gamma (IFN-γ), interleukin-1 (IL-1), IL-2, IL-3, IL-6, IL-7, IL-12, IL-15, tumor necrosis factor-alpha (TNF-α), granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), or other cytokine. In this regard, generating an immune response may comprise a shift from a Th2 type response to a Th1 type response or in certain embodiments a shift from a Th1 type response to a Th2 type response. In other embodiments, generating an immune response may comprise the stimulation of a predominantly Th1 or a Th2 type response.

In a further embodiment, generating an immune response comprises an increase in target-specific antibody production of between 1.5 and 5 fold in a subject administered the adenovirus vectors of the present invention as compared to an appropriate control. In another embodiment, generating an immune response comprises an increase in target-specific antibody production of about 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 15, 16, 17, 18, 19, 20, or more fold in a subject administered the adenovirus vector as compared to a control.

Thus the present invention provides methods for generating an immune response against a target antigen of interest comprising administering to the individual an adenovirus vector comprising: a) a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding the target antigen; and readministering the adenovirus vector

at least once to the individual; thereby generating an immune response against the target antigen. In certain embodiments, the present invention provides methods wherein the vector administered is not a gutted vector.

In a further embodiment, the present invention provides methods for generating an immune response against a target antigen in an individual, wherein the individual has preexisting immunity to Ad, by administering to the individual an adenovirus vector comprising: a) a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding the target antigen; and readministering the adenovirus vector at least once to the individual; thereby generating an immune response against the target antigen.

With regard to preexisting immunity to Ad, this can be determined using methods known in the art, such as antibody-based assays to test for the presence of Ad antibodies. Further, in certain embodiments, the methods of the present invention include first determining that an individual has preexisting immunity to Ad then administering the E2b deleted adenovirus vectors of the invention as described herein.

One embodiment of the invention provides a method of generating an immune response against one or more target antigens in an individual comprising administering to the individual a first adenovirus vector comprising a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and a nucleic acid encoding at least one target antigen; administering to the individual a second adenovirus vector comprising a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and a nucleic acid encoding at least one target antigen, wherein the at least one target antigen of the second adenovirus vector is the same or different from the at least one target antigen of the first adenovirus vector.

Thus, the present invention contemplates multiple immunizations with the same E2b deleted adenovirus vector or multiple immunizations with different E2b deleted adenovirus vectors. In each case, the adenovirus vectors may comprise nucleic acid sequences that encode one or more target antigens as described

elsewhere herein. In certain embodiments, the methods comprise multiple immunizations with an E2b deleted adenovirus encoding one target antigen, and re-administration of the same adenovirus vector multiple times, thereby inducing an immune response against the target antigen.

In a further embodiment, the methods comprise immunization with a first adenovirus vector that encodes one or more target antigens, and then administration with a second adenovirus vector that encodes one or more target antigens that may be the same or different from those antigens encoded by the first adenovirus vector. In this regard, one of the encoded target antigens may be different or all of the encoded antigens may be different, or some may be the same and some may be different. Further, in certain embodiments, the methods include administering the first adenovirus vector multiple times and administering the second adenovirus multiple times. In this regard, the methods comprise administering the first adenovirus vector 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more times and administering the second adenovirus vector 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more times. The order of administration may comprise administering the first adenovirus one or multiple times in a row followed by administering the second adenovirus vector one or multiple times in a row. In certain embodiments, the methods include alternating administration of the first and the second adenovirus vectors as one administration each, two administrations each, three administrations each, and so on. In certain embodiments, the first and the second adenovirus vectors are administered simultaneously. In other embodiments, the first and the second adenovirus vectors are administered sequentially.

As would be readily understood by the skilled artisan, more than two adenovirus vectors may be used in the methods of the present invention. Three, 4, 5, 6, 7, 8, 9, 10 or more different adenovirus vectors may be used in the methods of the invention. In certain embodiments, the methods comprise administering more than one E2b deleted adenovirus vector at a time. In this regard, immune responses against multiple target antigens of interest can be generated by administering

multiple different adenovirus vectors simultaneously, each comprising nucleic acid sequences encoding one or more antigens.

The present invention provides methods of generating an immune response against any target antigen, such as those described elsewhere herein.

The present invention provides methods of generating an immune response against any infectious agent, such as those described elsewhere herein.

As noted elsewhere herein, the adenovirus vectors of the invention comprise nucleic acid sequences that encode one or more target antigens of interest from any one or more of the infectious agents against which an immune response is to be generated. For example, target antigens may include, but are not limited to, viral coat proteins, *i.e.*, influenza neuraminidase and hemagglutinin, HIV gp160, p24, gp120, gp41, envelope, protease, or reverse transcriptase, or derivatives of any of these viral proteins; SARS coat proteins, herpes virion proteins, WNV proteins, *etc.* Target antigens may also include bacterial surface proteins including pneumococcal PsaA, PspA, LytA, surface or virulence associated proteins of bacterial pathogens such as *Nisseria gonnorhea,* outer membrane proteins or surface proteases.

In certain embodiments, the adenovirus vectors are used to generate an immune response against a cancer. In this regard, the methods include generating an immune response against carcinomas or sarcomas such as solid tumors, lymphomas or leukemias. Thus, the adenovirus vectors described herein are used to generate an immune response against a cancer including but not limited to carcinomas or sarcomas such as neurologic cancers, melanoma, non-Hodgkin's lymphoma, Hodgkin's disease, leukemias, plasmocytomas, adenomas, gliomas, thymomas, breast cancer, prostate cancer, colo-rectal cancer, kidney cancer, renal cell carcinoma, uterine cancer, pancreatic cancer, esophageal cancer, lung cancer, ovarian cancer, cervical cancer, testicular cancer, gastric cancer, multiple myeloma, hepatoma, acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), and chronic lymphocytic leukemia (CLL), or other cancers.

Further, in this regard, the cancer target antigens may include but are not limited to antigens derived from a variety of tumor proteins. Illustrative tumor proteins useful in the present invention include, but are not limited to any one or more of, p53, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, BAGE, DAM-6, -10, GAGE-1, -2, -8, GAGE-3, -4, -5, -6, -7B, NA88-A, NY-ESO-1, MART-1, MC1R, Gp100, PSA, PSM, Tyrosinase, TRP-1, TRP-2, ART-4, CAMEL, CEA, Cyp-B, Her2/neu, hTERT, hTRT, iCE, MUC1, MUC2, PRAME, P15, RU1, RU2, SART-1, SART-3, WT1, AFP, β-catenin/m, Caspase-8/m, CEA, CDK-4/m, ELF2M, GnT-V, G250, HSP70-2M, HST-2, KIAA0205, MUM-1, MUM-2, MUM-3, Myosin/m, RAGE, SART-2, TRP-2/INT2, 707-AP, Annexin II, CDC27/m, TPI/mbcr-abl, ETV6/AML, LDLR/FUT, Pml/RARα, and TEL/AML1. These and other tumor proteins are known to the skilled artisan.

Methods are also provided for treating or ameliorating the symptoms of any of the infectious diseases or cancers as described herein. The methods of treatment comprise administering the adenovirus vectors one or more times to individuals suffering from or at risk from suffering from an infectious disease or cancer as described herein. As such, the present invention provides methods for vaccinating against infectious diseases or cancers in individuals who are at risk of developing such a disease. Individuals at risk may be individuals who may be exposed to an infectious agent at some time or have been previously exposed but do not yet have symptoms of infection or individuals having a genetic predisposition to developing a cancer or being particularly susceptible to an infectious agent.

The present invention contemplates the use of adenovirus vectors for the *in vivo* delivery of nucleic acids encoding a target antigen. Once injected into a subject, the nucleic acid sequence is expressed resulting in an immune response against the antigen coded for by the sequence. The adenovirus vector vaccine is administered in an "effective amount", that is, an amount of adenovirus vector that is effective in a selected route or routes of administration to elicit an immune response as described elsewhere herein. In certain embodiments, an effective amount is one that induces an immune response effective to facilitate protection or treatment of the

host against the target infectious agent or cancer. The amount of vector in each vaccine dose is selected as an amount which induces an immune, immunoprotective or other immunotherapeutic response without significant adverse effects generally associated with typical vaccines. Once vaccinated, subjects may be monitored to determine the efficacy of the vaccine treatment. Monitoring the efficacy of vaccination may be performed by any method known to a person of ordinary skill in the art. In some embodiments, blood or fluid samples may be assayed to detect levels of antibodies. In other embodiments, ELISpot assays may be performed to detect a cell-mediated immune response from circulating blood cells or from lymphoid tissue cells.

The adenovirus vectors of the invention are generally prepared as known in the art (see *e.g.*, Hodges *et al.*, 2000 *supra;* or Amalfitano *et al.*, 1998 *supra*). For example, in certain embodiments, tissue culture plates containing E.C7 or C-7 cells are infected with the adenovirus vector virus stocks at an appropriate MOI (*e.g.*, 5) and incubated at 37.0°C for 40 h. The infected cells are harvested, resuspended in an appropriate buffer such as 10 mM Tris-Cl (pH 8.0), and sonicated, and the virus is purified by two rounds of cesium chloride density centrifugation. In certain techniques, the virus containing band is desalted over a Sephadex CL-6B column (Pharmacia Biotech, Piscataway, N.J.), glycerol is added to a concentration of 12%, and aliquots are stored at -80°C. The titer of the stock is measured (*e.g.*, by measurement of the optical density at 260 nm of an aliquot of the virus after SDS lysis). GMP procedures for producing appropriate Ad stocks for human administration are used where appropriate.

For administration, the adenovirus vector stock is combined with an appropriate buffer, physiologically acceptable carrier, excipient or the like. In certain embodiments, an appropriate number of adenovirus vector particles are administered in an appropriate buffer, such as, sterile PBS. In certain circumstances it will be desirable to deliver the adenovirus vector compositions disclosed herein parenterally, intravenously, intramuscularly, or even intraperitoneally. In certain embodiments, solutions of the active compounds as free base or pharmacologically

acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. In other embodiments, E2b deleted adenovirus vectors may be delivered in pill form, delivered by swallowing or by suppository.

Illustrative pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (for example, see U. S. Patent 5,466,468). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria, molds and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.,* glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants. The prevention of the action of microorganisms can be facilitated by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

In one embodiment, for parenteral administration in an aqueous solution, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl

solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. Moreover, for human administration, preparations will of course preferably meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

The carriers can further comprise any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

In certain embodiments, the adenovirus vectors of the invention may be administered in conjunction with one or more immunostimulants, such as an adjuvant. An immunostimulant refers to essentially any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an antigen. One type of immunostimulant comprises an adjuvant. Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated

sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, may also be used as adjuvants.

Within certain embodiments of the invention, the adjuvant composition is preferably one that induces an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (*e.g.,* IFN-γ, TNFα, IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (*e.g.,* IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a patient will support an immune response that includes Th1- and Th2-type responses. Within certain embodiments, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, *Ann. Rev. Immunol. 7*:145-173, 1989.

Certain illustrative adjuvants for eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with an aluminum salt. MPL® adjuvants are available from GlaxoSmithKlein (Research Triangle Park, NC; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato *et al.*, *Science 273*:352, 1996. Another adjuvant for use in the present invention comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins . Other formulations may include more than one saponin in the adjuvant combinations of the present invention,

for example combinations of at least two of the following group comprising QS21, QS7, Quil A, β-escin, or digitonin.

In certain embodiments, the compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. The delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, J Controlled Release 1998 Mar 2;52(1-2):81-7) and lysophosphatidyl-glycerol compounds (U. S. Patent 5,725,871) are well-known in the pharmaceutical arts. Likewise, illustrative transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U. S. Patent 5,780,045.

In certain embodiments, liposomes, nanocapsules, microparticles, lipid particles, vesicles, and the like, are used for the introduction of the compositions of the present invention into suitable host cells/organisms. In particular, the compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like. Alternatively, compositions of the present invention can be bound, either covalently or non-covalently, to the surface of such carrier vehicles.

The formation and use of liposome and liposome-like preparations as potential drug carriers is generally known to those of skill in the art (see for example, Lasic, Trends Biotechnol 1998 Jul;16(7):307-21; Takakura, Nippon Rinsho 1998 Mar;56(3):691-5; Chandran *et al.*, Indian J Exp Biol. 1997 Aug;35(8):801-9; Margalit, Crit Rev Ther Drug Carrier Syst. 1995;12(2-3):233-61; U.S. Patent 5,567,434; U.S. Patent 5,552,157; U.S. Patent 5,565,213; U.S. Patent 5,738,868 and U.S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally difficult to transfect by other procedures, including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.,* J Biol Chem. 1990 Sep 25; 265(27):16337-42; Muller *et al.*, DNA Cell Biol. 1990 Apr;9(3):221-9). Liposomes have been used effectively to introduce genes, various drugs, radiotherapeutic agents, enzymes, viruses, transcription factors, allosteric effectors and the like, into a variety of cultured cell lines and animals. Furthermore,

the use of liposomes does not appear to be associated with autoimmune responses or unacceptable toxicity after systemic delivery.

In certain embodiments, liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs).

Alternatively, in other embodiments, the invention provides for pharmaceutically-acceptable nanocapsule formulations of the compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (see, for example, Quintanar-Guerrero *et al.*, Drug Dev Ind Pharm. 1998 Dec;24(12):1113-28). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) may be designed using polymers able to be degraded *in vivo*. Such particles can be made as described, for example, by Couvreur *et al.,* Crit Rev Ther Drug Carrier Syst. 1988;5(1):1-20; zur Muhlen *et al.*, Eur J Pharm Biopharm. 1998 Mar; 45(2):149-55; Zambaux *et al.* J Controlled Release. 1998 Jan 2; 50(1-3):31-40; and U. S. Patent 5,145,684.

Routes and frequency of administration of the therapeutic compositions described herein, as well as dosage, will vary from individual to individual, and from disease to disease, and may be readily established using standard techniques. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration), in pill form (*e.g.* swallowing, suppository for vaginal or rectal delivery). In certain embodiments, between 1 and 10 doses may be administered over a 52 week period. In certain embodiments, 6 doses are administered, at intervals of 1 month, and further booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. As such, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more doses may be administered over a 1 year period or over shorter or longer periods, such as over 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 week periods. Doses may be administered at 1, 2, 3, 4, 5, or 6 week intervals or longer intervals.

A suitable dose is an amount of an adenovirus vector that, when administered as described above, is capable of promoting a target antigen immune response as described elsewhere herein. In certain embodiments, the immune response is at least 10-50% above the basal (*i.e.,* untreated) level. Such response can be monitored by measuring the target antigen(s) antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing patient tumor or infected cells *in vitro,* or other methods known in the art for monitoring immune responses. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome of the disease in question in vaccinated patients as compared to non-vaccinated patients.

In general, an appropriate dosage and treatment regimen provides the adenovirus vectors in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome for the particular disease being treated in treated patients as compared to non-treated patients. Such improvements in clinical outcome would be readily recognized by a treating physician. Increases in preexisting immune responses to a target protein generally correlate with an improved clinical outcome. Such immune responses may generally be evaluated using standard proliferation, cytotoxicity or cytokine assays, which may be performed using samples obtained from a patient before and after treatment.

While one advantage of the present invention is the capability to administer multiple vaccinations with the same or different adenovirus vectors, particularly in individuals with preexisting immunity to Ad, the adenoviral vaccines of this invention may also be administered as part of a prime and boost regimen. A mixed modality priming and booster inoculation scheme may result in an enhanced immune response. Thus, one aspect of this invention is a method of priming a subject with a plasmid vaccine, such as a plasmid vector comprising a target antigen of interest, by administering the plasmid vaccine at least one time, allowing a predetermined length of time to pass, and then boosting by administering the adenovirus vector. Multiple primings, *e.g.*, 1-4, may be employed, although more

may be used. The length of time between priming and boost may typically vary from about four months to a year, but other time frames may be used. In certain embodiments, subjects may be primed four times with plasmid vaccines, and then boosted 4 months later with the adenovirus vector.

EXAMPLES

EXAMPLE 1

MULTIPLE INJECTIONS OF AD5NULL ADENOVIRUS VECTOR PRODUCES ANTI-ADENOVIRUS ANTIBODIES

This example shows that multiple injections of Ad5-null results in the production of anti-adenovirus antibodies in the injected subjects

It was demonstrated that the Ad5Null adenovirus vector that does not contain any heterologous nucleic acid sequences, generates a neutralizing immune response in mice. In particular, in one experiment, female Balb/c mice aged 5-7 weeks were immunized with Ad5Null viral particles at 14 day intervals. To determine the presence of anti-adenovirus antibodies, an enzyme linked immunosorbent assay (ELISA) was used. For this ELISA, 10^9 viral particles were coated onto microtiter wells in 100 µL of 0.05M carbonate/bicarbonate buffer, pH 9.6, and incubated overnight at room temperature. For a standard immunoglobulin G (IgG) reference curve, 200 ng, 100 ng, 50 ng, 25 ng, and 0 ng of purified mouse IgG (Sigma Chemicals) were coated onto microtiter wells as described above. After incubation, all wells were washed 3 times with 250 µL of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.4. After washing, 250 µL of BSA/PBS was added to all and incubated for 30 minutes at room temperature to block unbound sites. After incubation, all wells were washed 3 times with 250 µL of BSA/PBS. After washing, 200 µL of a 1/100 serum dilution in BSA/PBS was added to wells and incubated for 1 hour at room temperature. For a positive control, 200 µL of a 1/10000 dilution of anti-adenovirus antiserum (Biodesign International) in BSA/PBS were added to wells. Control wells contained BSA/PBS only. After incubation, all wells were washed 3 times with 250 µL of BSA/PBS. After washing, 200 µL of a 1/10000 dilution of peroxidase conjugated gamma chain specific goat anti-mouse IgG (Sigma Chemicals) in BSA/PBS were added to each well and incubated for 1 hour at room temperature. After incubation, all wells were washed 3 times with 250 µL of

BSA/PBS. After washing, 200 µL of developing reagent (0.5 mg/mL 1,2 phenylenediamine in 0.2M potassium phosphate buffer, pH 5.0, containing 0.06% hydrogen peroxide) was added to each well and incubated for 30-40 minutes at room temperature. After incubation, the color reaction was stopped by addition of 50 µL 5N HCl to each well. All wells were then read in a microwell plate reader at 492 nm. After readings were obtained, the optical density readings of unknown samples were correlated with the standard IgG curve to obtain the nanograms of IgG bound per well. This was performed using the INSTAT statistical package.

As shown in Figure 1, significant levels ($P<0.001$) of anti-adenovirus IgG antibody were detected in mice 2 weeks after a first injection with 10^{10} Ad-5-null. A significantly higher level ($P<0.001$) was observed 2 weeks after a second injection with 10^{10} adenovirus. Significantly higher ($P<0.001$) levels of antibody were continued to be observed 2 weeks after a third injection with 10^{10} Ad5-null. Each value represents the average of triplicate determinations from pooled sera of 5 mice in each group. These results indicate that multiple injections of Ad5-null results in the production of anti-adenovirus antibodies in the injected subjects.

To determine the presence of neutralizing antibody to Ad, the following assay was utilized. A HEK-293T cell line was cultured in 200 µL of culture medium consisting of DMEM containing 10% fetal calf serum (DMEM/FCS) in microwell tissue culture plates at a cell concentration of 2×10^3 cells per well for 24 hours at 37 C in 5% CO_2. After incubation, 100 µL of culture medium was removed from triplicate wells and mixed with 20 µL of DMEM/FCS containing viral particles (VP). After mixing, the 120 µL mixture was added back to the respective microwells. In another set of triplicate wells, 100 µL of culture medium was removed and mixed with 20 µL of heat inactivated (56 C for 1 hour) Ad immune mouse serum previously incubated with VP for one hour at room temperature. After mixing, the 120 µL mixture was added back to the respective wells. In triplicate cell control wells, 20 µL of DMEM/FCS was added to control for total culture medium volume. Triplicate medium only control wells contained 220 µL of DMEM/FCS. The tissue culture plate was incubated for an additional 3 days at 37 C in 5% CO_2. After incubation, 40 µL of

PROMEGA cell viability reagent (Owen's reagent) was added to all wells and incubated for 75 minutes at 37C in 5% CO_2. In this assay, the Owen's reagent (MTS tetrazolium compound) is bioreduced by viable cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by absorbance at 490 nm is directly proportional to the number of living cells in culture. After incubation, 150 µL was removed from each well and transferred to another microwell plate for optical density readings. Optical density readings at 492 nm were subsequently obtained using a microwell plate reader.

In an experiment to detect the presence of neutralizing antibodies to Ad, groups of 5 mice each were injected once, twice, or three times with 10^{10} Ad5null at two week intervals. Two weeks after the final injection of virus, mice were bled, pooled, and assessed for neutralizing antibody as described above using 4 x 10^7 VP incubated with or without heat inactivated sera. Cells cultured alone served as a control group. As shown in Figure 2, normal mice and mice injected one time with Ad5null did not exhibit significant levels of neutralizing antibody. Mice injected two times with Ad exhibited significant ($P<0.05$) levels of neutralizing antibody as compared with cells incubated with virus only. Mice injected three times with Ad5-null also exhibited significant ($P<0.01$) levels of neutralizing antibody as compared with cells incubated with virus only.

EXAMPLE 2

MULTIPLE INJECTIONS OF AN E2B DELETED ADENOVIRUS VECTOR GENERATES AN IMMUNE RESPONSE AGAINST TARGET ANTIGENS

This example shows that multiple injections of an E2b deleted adenovirus vector containing HIV-gag results in the production of HIV-gag immunity.

Two groups of mice were used for this experiment. One group served as a normal control group. The second group was injected 3 times with E2b deleted Ad containing HIV-gag at 2 week intervals. Four weeks after the last injection, mice were bled and assessed for IgG antibody levels using an ELISA assay as follows: For this assay, 100 ng of a purified mixture of HIV-gag proteins p17/p24 were coated

onto microtiter wells in 100 µL of 0.05M carbonate/bicarbonate buffer, pH 9.6, and incubated overnight at room temperature. For a standard IgG reference curve, purified mouse IgG (SigmaChemicals) in quantities of 200 ng, 100 ng, 50 ng, 25 ng, and 0 ng were coated onto microtiter wells as described above. After incubation, all wells were washed 3 times with 250 µL of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.4. After washing, 200 µL of BSA/PBS was added to wells and incubated for 30 minutes at room temperature to block any remaining sites in the microtiter wells. After incubation, all wells were washed 3 times with 250 µL of BSA/PBS. After washing, 200 µL of a 1/100 dilution of mouse serum in BSA/PBS was added to wells and incubated for one hour at room temperature. For a positive control, 100 ng of mouse monoclonal anti-p24 IgG antibody in BSA/PBS was added to wells. Blank control wells contained BSA/PBS only. After incubation, all wells were washed 3 times with 250 µL of BSA/PBS. After washing, 200 µL of a 1/10000 dilution of peroxidase conjugated gamma chain specific goat anti-mouse IgG (Sigma Chemicals) in BSA/PBS was added to each well and incubated for one hour at room temperature. After incubation, all wells were washed 3 times with 250µL of BSA/PBS. After washing, 200 µL of developing reagent (0.5 mg/mL 1,2 phenylenediamine in 0.2M potassium phosphate buffer, pH 5.0, containing 0.06% hydrogen peroxide) was added to each well and incubated for 30-40 minutes at room temperature. After incubation, the color reaction was stopped by addition of 50µL 5M HCl to each well. All wells were read in a microwell plate reader at 492 nm. After readings were obtained, the optical density readings of unknown samples were correlated with the standard IgG curve to obtain nanograms of IgG bound per well. This was performed utilizing the INSTAT statistical package.

As shown in Figures 3, low but significant levels of Gag IgG were detected in mice 2 weeks after a second injection and 4 weeks after a third injection with 10^{10} E2b deleted Ad containing the HIV-gag gene. Moreover, when compared with respective pre-injections bleeds, low but significant levels ($P<0.01$) of detectable antibody were observed up to 8 weeks (Day 84) as well as 13 weeks (Day 119) post 3rd injection with E2b deleted Ad-gag vector vaccine.

EXAMPLE 3

MULTIPLE INJECTIONS OF AN E2B DELETED ADENOVIRUS VECTOR GENERATES AN IMMUNE RESPONSE AGAINST MULTIPLE TARGET ANTIGENS

This example demonstrates that mice injected multiple times with an E2b deleted adenovirus vector vaccine expressing a first target antigen (HIV-gag) and subsequently injected multiple times with an E2b deleted adenovirus vector expressing a second target antigen (β-galactosidase) produce an immune response against the first and the second antigen.

A group of five mice were injected 3 times at 2 week intervals with 10^{10} E2b deleted adenovirus vector vaccine containing the HIV-gag gene. Four weeks later, the mice were injected two times at a weekly interval with 10^{10} E2b deleted adenovirus vector vaccine containing β-galactosidase. A group of 5 mice served as a normal control group. Sera from mice injected only with E2b deleted Ad-βgal that showed high levels of β-galactosidase IgG antibody served as a positive control.

To determine the presence of β-galactosidase antibodies, an enzyme linked immunosorbent assay (ELISA) was used. For this ELISA, 100ng of purified β-galactosidase was coated onto microtiter wells in 100 µL of 0.05M carbonate/bicarbonate buffer, pH 9.6 and incubated over night at room temperature. For a standard IgG reference curve, purified mouse IgG (Sigma Chemicals) in quantities of 200 ng, 100 ng, 50 ng, 25 ng, and 0 ng were coated onto microtiter wells as described above. After incubation, all wells were washed 3 times with 250 µL of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.4. After washing, 250 µL of BSA/PBS was added to all wells and incubated for 30 minutes at room temperature. After incubation, all were washed 3 times with 250 µL of BSA/PBS. After washing, 200 µL of a 1/100 of serum in BSA/PBS was added to wells and incubated for 1 hour at room temperature. Blank control wells contained BSA/PBS only. After incubation, all wells were washed 3 times with 250 µL of BSA/PBS. After washing, 200 µL of a 1/10000 dilution of peroxidase conjugated gamma chain specific goat anti-mouse IgG (Sigma Chemicals) in BSA/PBS was added to each well and incubated for 1 hour at room temperature. After incubation,

all wells were washed 3 times with 250 µL of BSA/PBS. After washing, 200 µL of developing reagent (0.5 mg/mL 1, 2 phenylenediamine in 0.2M potassium phosphate buffer, pH 5.0, containing 0.06% hydrogen peroxide) was added to each well and incubated for 30-40 minutes at room temperature. After incubation, the color reaction was stopped by addition of 50 µL 5N HCl to each well. All wells were then read in a microwell plate reader at 492 nm. After readings were obtained, the optical density readings of unknown samples were correlated with the standard IgG curve to obtain the nanograms of IgG bound per well. This was performed utilizing the INSTAT statistical package.

As shown in Figure 4, sera assessed 2 weeks after the last injection from mice injected with E2b deleted adenovirus vector containing *E. coli* β-galactosidase exhibited significantly ($P<0.01$) higher levels of anti-β-galactosidase IgG antibodies as compared to normal control mice. Detectable levels of anti-β-galactosidase antibody persisted up to 5 weeks after the last injection. Moreover, significant levels of HIV-gag immunity were still readily detected and levels of these antibodies even increased.

In a related experiment, cellular immune responses in mice were assessed after multiple immunizations. Mice were immunized with E2b deleted adenovirus vector vaccine containing the HIV-gag gene three times at 14 day intervals. Four weeks later, the mice were subsequently immunized with E2b deleted adenovirus vector vaccine containing the β-galactosidase gene twice at 14 day intervals. ELISpot assays were performed to determine cellular mediated immune responses in vaccinated subjects. ELISpot assay kits were obtained from eBioscience and assay plates were prepared according to manufacturer's specifications. The assay was performed as described in the manufacturer's instructions. Briefly, capture antibody was coated onto ELISpot assay plates per instructions and coated overnight. After washing and blocking unbound sites, mitogen, specific antigen, and controls were added to wells in complete RPMI-1640 culture medium at 100 µL per well. Spleen cells from mice were harvested and prepared for cell culture. Cells were then added to wells in a 100 µL volume at the

desired cell density. The ELISpot plates were then incubated at 37 C in a 5% CO_2 humidified incubator for approximately 48 hours. After incubation, the ELISpot plates were developed according to manufacturer's instructions. The data was expressed as the number of spot forming cells (SPC) per 10^6 splenocytes.

As shown in Figure 5A, ELISpot analysis showed that mice exhibited the production interferon-γ (IFN-γ) upon re-stimulation to HIV-gag and Ad5null virions. Furthermore, as shown in Figure 5B, ELISpot analysis also showed the production of interleukin-2 (IL-2) upon re-stimulation with HIV-gag, β-galactosidase, and Ad5null virions. These results indicated that cell-mediated immune responses could be generated in the same subjects after multiple injections with E2b deleted adenovirus vector vaccines encoding 2 differing target antigens. Moreover, the cell-mediated immune response against the second immunizing antigen occurred in the presence of immunity to Ad.

Thus, the E2b deleted adenovirus vectors of the present invention can be used to immunize against multiple antigens using regimens of multiple immunizations.

EXAMPLE 4

CELL MEDIATED IMMUNE (CMI) RESPONSE INDUCTION IN ADENOVIRUS 5 IMMUNE CYNOMOLGUS MACAQUES

This example shows that multiple injections of an E2b deleted adenovirus vector containing HIV-gag results in the production of HIV-gag immunity, even in the presence of Ad5 immunity.

Three non-human primates (NHP) were injected with a single dose of 10^{10} VP viable wild type Ad5. Ad5 neutralizing antibody (NAb) was measured 30 days after administration, and the NHP titers were ≥1:50 (Figure 6). The Ad5 immune NHP were then immunized three times; days 0, 27, and 58; with Ad5 [E1-, E2b-]-gag (10^{10} VP/dose). Peripheral blood mononuclear cells (PBMC) from individual NHP were collected at the indicated time points. CMI responses upon re-stimulation with HIV-gag protein were assayed 32 days after the final immunization

with Ad5 [E1-, E2b-]-gag. ELISpot analysis indicated that PBMC from all three NHP responded similarly upon re-stimulation with HIV-gag protein with an average frequency of 223 SFC/10^6 PBMC producing INF-γ and 207 SFC/10^6 PBMC producing IL-2 (Figure 7A and Figure 7B, respectively). These values were significantly ($P<0.05$) elevated when compared to their baseline values. The Ad5 viral NAb titers ranged from 1/1000 to 1/20,000 at the termination of this study (Figure 6).

As evidenced by the induction of specific CMI responses to the HIV-gag protein, these results indicate that NHP can be successfully immunized with the Ad5 [E1-, E2b-] vector platform in the presence of Adenovirus 5 immunity.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

What is claimed is:

1. A method of generating an immune response against one or more target antigens in an individual comprising:

administering to the individual an adenovirus vector comprising: a) a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding the one or more target antigens; and

readministering the adenovirus vector at least once to the individual;

thereby generating an immune response against the one or more target antigens.

2. A method for generating an immune response against one or more target antigens in an individual, wherein the individual has preexisting immunity to adenovirus, comprising:

administering to the individual an adenovirus vector comprising: a) a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding the one or more target antigens; and

readministering the adenovirus vector at least once to the individual;

thereby generating an immune response against the one or more target antigens.

3. The method of claim 1 or 2 wherein the target antigen comprises an HIV protein, herpes simplex virus protein, hepatitis C virus protein, malaria protein, plague protein, *M. tuberculosis protein,* or a *Streptococcus pneumonia protein,* or an immunogenic fragment thereof.

4. The method of claim 3, wherein the HIV protein is an HIV-gag protein.

5. The method of claim 1 or 2 wherein the target antigen comprises and antigen derived from a Venezuelan Equine Encephalitis Virus (VEEV), Western Equine Encephalitis Virus, or Japanese Encephalitis Virus protein.

6. The method of claim 1 or 2 wherein the target antigen comprises a Leishmania protein.

7. The method of claim 1 or 2 wherein the target antigen comprises a cancer protein such as carcinoembryonic antigen, Her2/Neu, WT-1.

8. The method of claim 1 or 2 wherein the target antigen comprises an influenza virus protein.

9. The method of claim 8 wherein the influenza virus protein is derived from the H5N1 influenza virus.

10. The method of claim 8 wherein the influenza virus protein is derived from an influenza virus selected from the group consisting of, H3N2, H9N1, H1N1, H2N2, H7N7, H1N2, H9N2, H7N2, H7N3, and H10N7.

11. The method of claim 8 wherein the influenza virus protein is haemagglutinin.

12. The method of claim 8 wherein the influenza virus protein is neuraminidase.

13. The method of claim 8 wherein the influenza virus protein is matrix protein M1.

14. A method of generating an immune response against one or more target antigens in an individual comprising:

administering to the individual a first adenovirus vector comprising: a) a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding at least one target antigen;

administering to the individual a second adenovirus vector comprising: a) a replication defective adenovirus vector, wherein the adenovirus vector has a deletion in the E2b region, and b) a nucleic acid encoding at least one target antigen, wherein the at least one target antigen of the second adenovirus vector is the same or different from the at least one target antigen of the first adenovirus vector;

thereby generating an immune response against one or more target antigens.

15. The method of any one of claims 1, 2, or 14 wherein the adenovirus vector is not a gutted vector.

16. The method of claim 14 wherein the individual has preexisting immunity to adenovirus.

17. The method of claim 14 wherein the at least one target antigen of the first and the second adenovirus vectors are derived from the same infectious organism.

18. The method of claim 14 wherein the at least one target antigen of the first and the second adenovirus vectors are derived from different infectious organisms.

19. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises an HIV protein.

20. The method of claim 19 wherein the at least one target antigen of the second adenovirus vector comprises an *E. coli* β-galactosidase.

21. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises an HIV protein and the at least one target antigen of the second adenovirus vector comprises an HIV protein.

22. The method of claim 21 wherein the at least one target antigen of the first adenovirus vector comprises an HIV protein and the at least one target antigen of the second adenovirus vector comprises an HIV protein that is different from the HIV protein of the first adenovirus vector.

23. The method of any one of claims 19-22 wherein the HIV protein is an HIV-gag protein.

24. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises a cancer protein.

25. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises a Her2/neu antigen.

26. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises a carcinoembryonic protein.

27. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises a bacterial antigen.

28. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises a viral antigen.

29. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises an antigen derived from a protozoan protein.

30. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises an antigen derived from a fungal protein.

31. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises an antigen derived from a mold protein.

32. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises an antigen derived from mammalian protein.

33. The method of claim 14 wherein the at least one target antigen of the first adenovirus vector comprises an antigen derived from an avian protein.

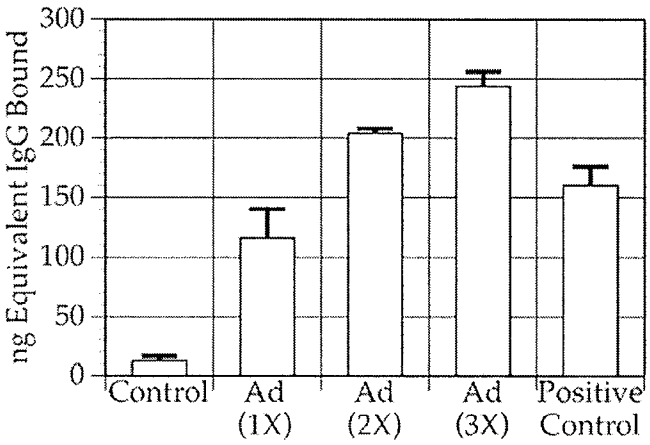
ng Equivalent IgG Bound
300
250
200
150
100
50
0
Control
Ad (1X)
Ad (2X)
Ad (3X)
Positive Control

FIG. 1

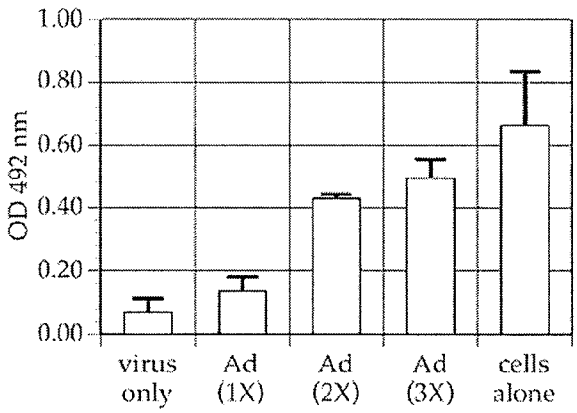
1.00
0.80
0.60
0.40
0.20
0.00
OD 492 nm
virus only
Ad (1X)
Ad (2X)
Ad (3X)
cells alone

FIG. 2

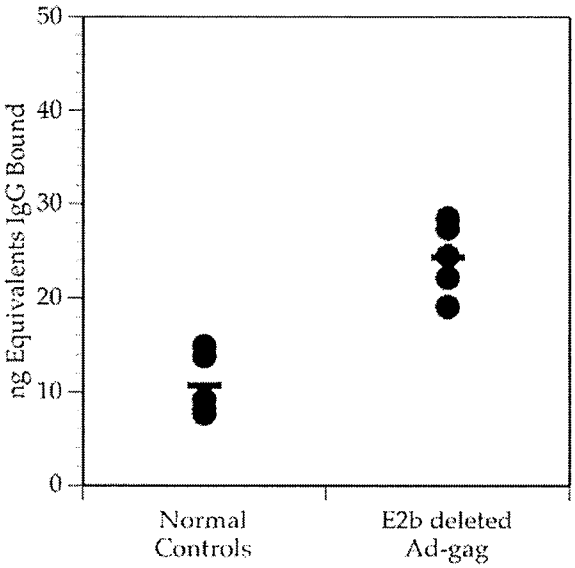
50
40
30
20
10
0
ng Equivalents IgG Bound
Normal Controls
E2b deleted Ad-gag

FIG. 3

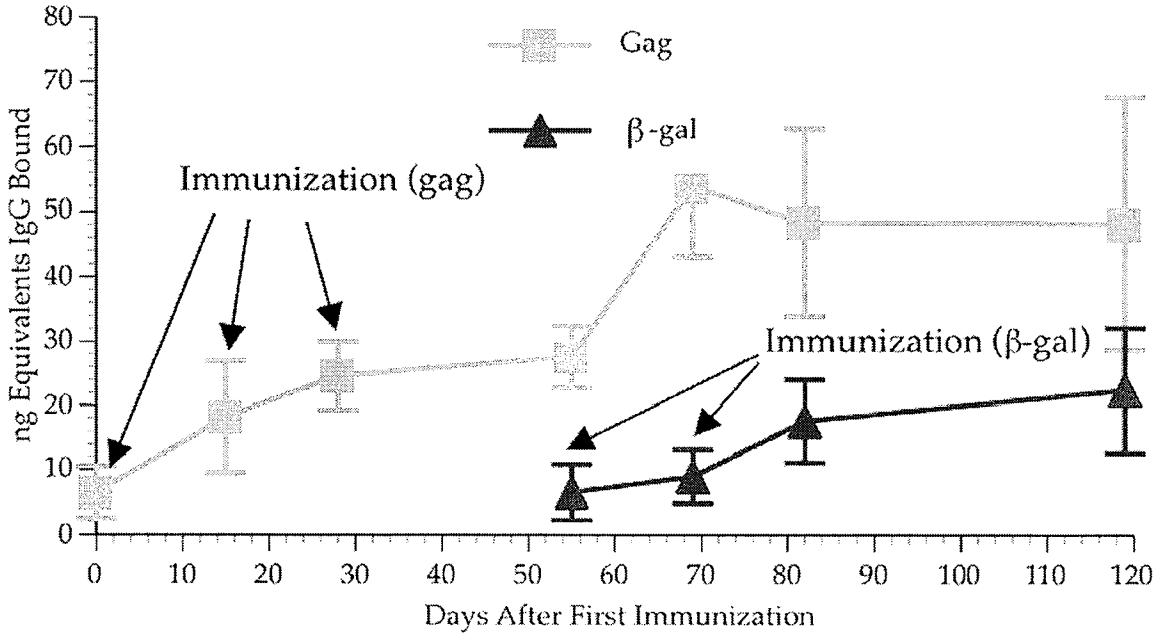
Gag
β-gal
Immunization (gag)
Immunization (β-gal)
ng Equivalents IgG Bound
Days After First Immunization
0
10
20
30
40
50
60
70
80
90
100
110
120

FIG. 4

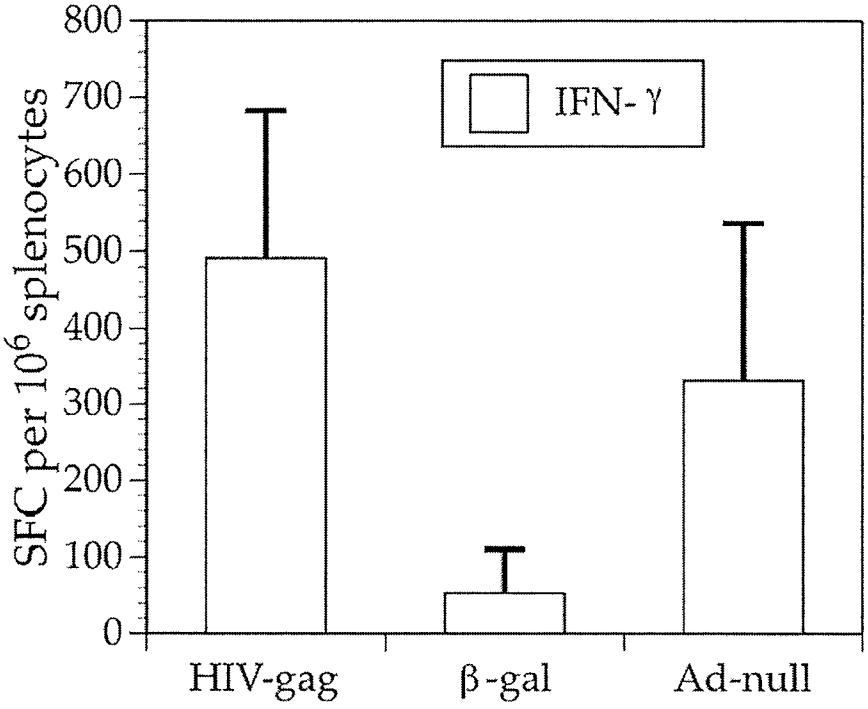
800
700
600
500
400
300
200
100
0
SFC per 10^6 splenocytes
IFN-γ
HIV-gag
β-gal
Ad-null

FIG. 5A

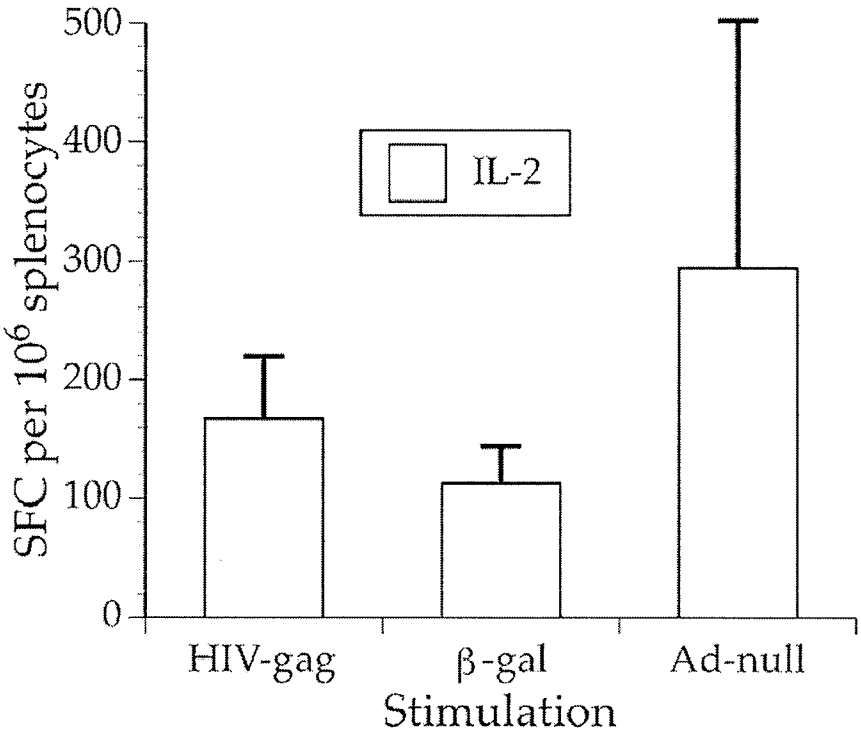
500
400
300
200
100
0
SFC per 10^6 splenocytes
IL-2
HIV-gag
β-gal
Ad-null
Stimulation

FIG. 5B

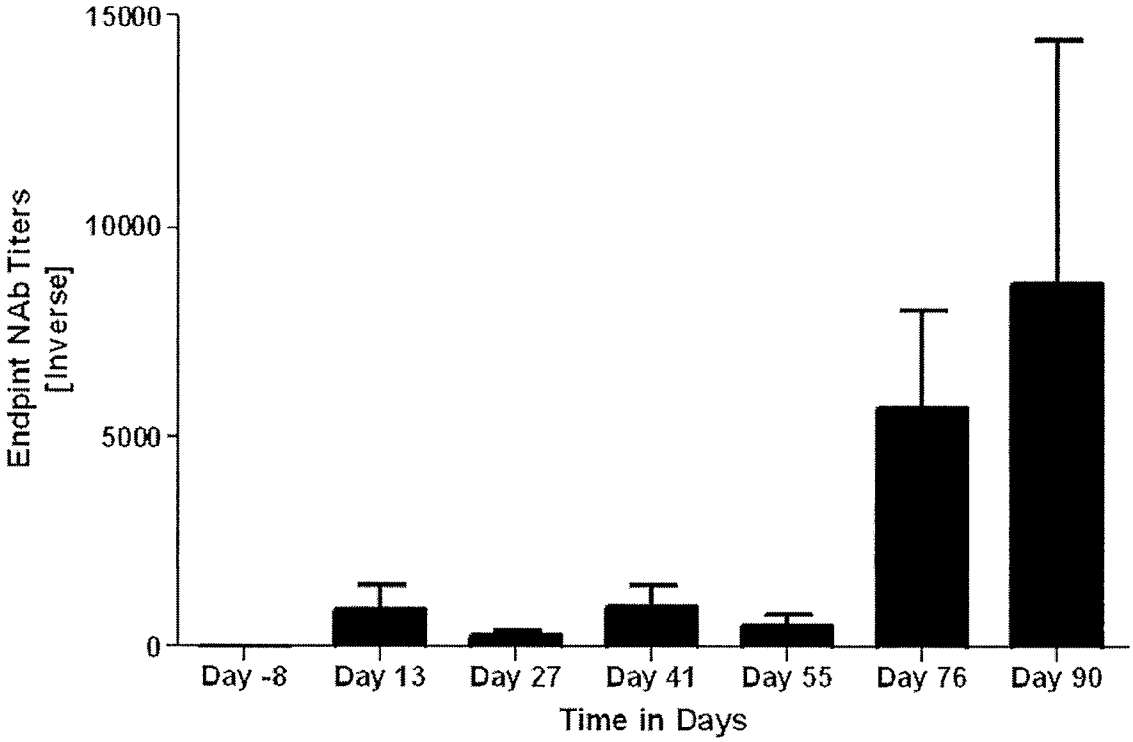
15000
10000
5000
0
Endpint NAb Titers
[Inverse]
Day -8
Day 13
Day 27
Day 41
Day 55
Day 76
Day 90
Time in Days

FIG. 6

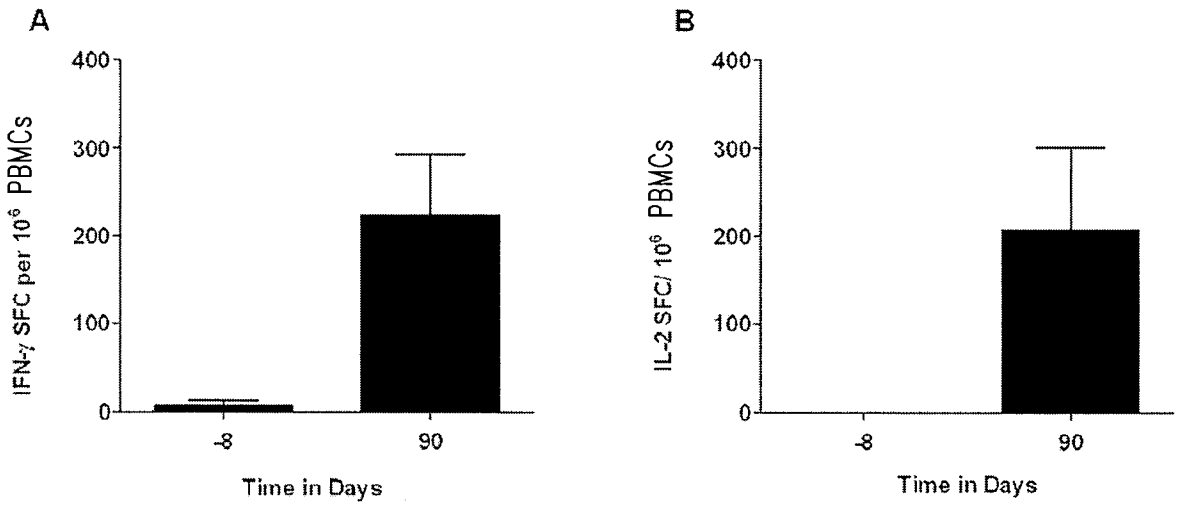
A
400
300
200
100
0
IFN-γ SFC per 10^6 PBMCs
-8
90
Time in Days
B
400
300
200
100
0
IL-2 SFC/ 10^6 PBMCs
-8
90
Time in Days

FIG. 7