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(71) Applicants (for all designated States except US): **EAST CAROLINA UNIVERSITY** [US/US]; 210 Spilman Building, Greenville, NC 27858-4353 (US). **EMORY UNIVERSITY** [US/US]; 2009 Ridgewood Drive, Atlanta, GA 30322 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): **ROSS, Ted, M.** [US/US]; Room 5N/96E, Brody Building, Greenville, NC 27858 (US). **ROBINSON, Harriet, L.** [US/US]; 954 Gatewood Road, NE, Atlanta, GA 30329 (US).

(74) Agent: **MYERS BIGEL SIBLEY & SAJOVEC**; P.O.Box 37428, Raleigh, NC 27627 (US).

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(54) **Title:** MEASLES VACCINE VECTORS AND METHODS OF USE

(57) **Abstract:** The present invention provides a novel vaccine expressing a fusion of the measles hemagglutinin (H) protein and the complement component, C3d, to enhance the titers of neutralizing antibody. Plasmids were generated that expressed a secreted (s) form of H and the same form fused to three tandem copies of the murine homologue of C3d (sH-3C3d). Analysis of titers of the antibody raised in vaccinated mice indicated that groups vaccinated with the DNA construct expressing sH-3C3d had higher titers of anti-H antibodies compared to serum from mice vaccinated with DNA expressing sH only. In addition, sH-3C3d elicited higher neutralizing antibody titers that inhibited MV induced plaque formation.

5 # MEASLES VACCINE VECTORS AND METHODS OF USE

Related Applications

This application claims the benefit of United States provisional application serial no. 60/317,659, filed September 6, 2002, the disclosure of
10 which is incorporated by reference herein in its entirety.

Government Support

This invention was made with Government support under grant number R21AI51213. The Government has certain rights in this invention.

15

Field of the Invention

The present invention concerns vaccine methods, compositions and formulations, particularly for the treatment of measles.

20

Background of the Invention

Measles virus (MV), which most commonly strikes children, is the causative agent of a highly contagious disease that is generally transmitted by aerosolized secretions. Most children recover uneventfully from the illness, but serious complications can occur, including pneumonia and involvement of the
25 central nervous system (Griffin, D.E. and Bellini, W.J., 1996). MV has been controlled during the past four decades by immunization with live attenuated vaccines in Europe and North America, However, MV infection is still a major health problem in undeveloped countries, and is the highest cause of mortality among children from a vaccine-preventable disease in the world *(Mortality and*
30 *Morbidity Weekly Report,* 2000a). A need exists, therefore, for novel vaccines that provide antigen presenting cells with sufficient antigen and the correct inflammatory response to raise a strong immune response and which may be

safely used in animals, including humans, for eliciting immune response against infectious diseases like measles.

Summary of the Invention

5 The present invention provides a novel vaccine useful for eliciting a protective response in a host against infection with an infectious agent such as the measles virus.

The novel vaccine of the present invention can include a DNA transcription unit of any virus, bacteria, parasite and/or fungi as a DNA 10 immunogen. For example, one embodiment of the present invention provides a DNA-based vaccine expressing a fusion of the measles hemagglutinin (H) protein and the complement component, C3d, to enhance the titers of neutralizing antibody. However, other DNA immunogens such as influenza virus hemagglutinin (HA), measles nucleoprotein (NP) genes, HA genes from canine 15 distemper virus, and the like are contemplated to be within the scope of the present invention.

In an alternate embodiment of the vaccine of the present invention, plasmids were generated that expressed a secreted **(S)** form of H fused to three tandem copies of the murine homologue of C3d (sH-3C3d). However, other 20 immunogens like genetic adjuvants may also be included in embodiments of the present invention. For example, a genetic adjuvant such as a mutant caspase gene, an interferon regulatory factor, or the like may be included in the DNA vaccine construct to increase the immune response elicited by vaccine administration.

25 The present invention also provides a novel vaccine composition for use in eliciting an immune response in a patient against an infectious agent. For example, in one embodiment of the present invention, the vaccine composition comprises a DNA vaccine construct expressing a secreted form of the measles hemagglutinin (H) protein fused to tandem repeats of the complement 30 component, C3d, and a pharmaceutical carrier. Optionally, in yet another alternate embodiment of the present invention, the vaccine composition comprises, as one component, a measles DNA vaccine made according to the present invention and at least one other antigen, including other bacterial, viral,

protozoal or fungal antigens or antitoxins capable of inducing a protective immune response against infectious agents, in a pharmaceutically-acceptable carrier.

The present invention is also directed to a novel method of immunizing a 5 patient by administering a therapeutically effective amount of the novel DNA-based vaccine composition of the present invention in a therapeutically effective amount sufficient to provide an immune response in the vaccinated patient.

Additional objects and aspects of the present invention will become more apparent upon review of the detailed description set forth below when taken in 10 conjunction with the accompanying figures, which are briefly described as follows.

Brief Description of the Figures

Fig. 1 is a schematic representation of a vector DNA vaccine construct 15 made according to the present invention.

Fig. 1A: The pTR600 vector contains two copies of the cytomegalovirus immediate-early promoter (CMV-IE) plus intron A (IA) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation terminator (BGH poly A) for termination of transcription. The vector also contains the Col 20 E1 origin of replication for prokaryotic replication as well as the Kanamycin resistance *(Kanr)* gene for selection in antibiotic media. Measles H inserts were cloned into the multiple cloning region using the *Hind* III and *Bam* HI restriction endonuclease sites.

Fig. 1B: the top schematic represents the wild-type, transmembrane 25 form of the H protein. The middle schematic represents the secreted form of the H that was placed in frame with the tPA leader sequence (Yang et al., 1997). The bottom schematic represents the sH-3C3d construct used as a vaccine insert. Linkers composed of two repeats of 4 glycines and a serine {$(G_4S)_2$} were fused at the junctures of HA and C3d and between each C3d repeat. 30 Rectangles indicate domains. TM: transmembrane.

Fig. 2 is a blot showing the expression of vaccine constructs *in vitro.* Human embryonic kidney cells, 293T, were transfected with 2 µg of each vaccine plasmid. Supernatant was collected and 1.5% of total volume was

subjected to electrophoresis on a 10% polyacrylamide gel. Lane 1: sH-3C3d-DNA; Lane 2: pTR600 vector-DNA; and Lane 3: sH-DNA.

Fig. 3 shows anti-Measles virus H IgG responses as measured by ELISA in mice vaccinated with 0.1 μg of DNA. Sera were collected at indicated weeks post-prime. Sera were obtained from mice and the range of the serum titers for each individual mouse is indicated with sH **(●)** and sH-3C3d **(■).** Numbers in parenthesis indicate the number of mice with same titer of sera. The line graph represents the average titer from the collected sera of the individual mice per group at the indicated times with sH (○) and sH-3C3d (□). Background titers from mice vaccinated with vector only were subtracted from experimental values. Preimmune sera from mice had no detectable specific IgG.

Detailed Description of the Preferred Embodiments

Other objects, features and aspects of the present invention are disclosed in, or are obvious from, the following Detailed Description, one or more examples of which are illustrated in the accompanying drawings. It is to be understood by one of ordinary skill in the art that the present discussion is a description of exemplary embodiments only and is not intended as limiting the broader aspects of the present invention, which broader aspects are embodied in the exemplary constructions. In fact, it will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. For instance, features illustrated or described as part of one embodiment can be used in another embodiment to yield a still further embodiment. It is intended that the present invention cover such modifications and variations as come within the scope of the appended claims and their equivalents.

This description uses gene nomenclature accepted by the Cucurbit Genetics Cooperative as it appears in the *Cucurbit Genetics Cooperative Report* 18:85 (1995), herein incorporated by reference in its entirety. Using this gene nomenclature, genes are symbolized by italicized Roman letters. If a mutant gene is recessive to the normal type, then the symbol and name of the mutant gene appear in italicized lower case letters.

Throughout this application various publications are referenced. The disclosures of these publications are hereby incorporated by reference in their entireties in this application to more fully describe the state of the art to which this invention pertains.

5 This invention relates to novel DNA vaccine constructs, novel constructs expressing a fusion of measles H and the C3d component of complement, novel vaccine compositions comprising at least one DNA-expressed immunogen in a pharmaceutically acceptable carrier, and a novel method of immunizing patients, including animals and humans, against a pathogen by administering 10 the novel vaccine composition of the present invention. The novel immunization methods elicit both cell-mediated and humoral immune responses that may limit the infection, spread, or growth of the pathogen of interest and result in protection in the vaccinated host against subsequent challenge by the pathogen.

15 Classic references for DNA vaccines include the first demonstration of the raising of an immune response (Tang, De Vit and Johnston, 1992); the first demonstration of cytotoxic T-cell (Tc)-mediated immunity (Ulmer *et al.*, 1993); the first demonstration of the protective efficacy of intradermal (i.d.), intramuscular (i.m.), intravenous (i.v.), intranasal (i.n.), and gene gun (g.g.) 20 immunizations (Fynan *et al.*, 1993; Robinson, Hunt, and Webster, 1993); the first use of genetic adjuvants (Xiang and Ertl, 1995); the first use of library immunizations (Barry, Lai, and Johnston, 1995); and the first demonstration of the ability to modulate the T-helper type of an immune response by the method of DNA delivery (Feltquate *et al.*, 1997). A highly useful web site compiling DNA 25 vaccine information can be found at http://www.genweb.com/Dnavax/dnavax.html

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

30 *Definitions*

The term "animal" as used herein refers to all vertebrate animals, including humans and birds. The term also includes an individual animal in all stages of development, including embryonic and fetal stages.

The term “patient” as used herein refers to animals as well as humans.

The term “pathogen” as used herein refers to an infectious organism like a virus, bacteria, parasite, fungus, or the like which adversely affects a patient infected by the organism. If the adverse effect is serious enough, a disease state results from infection with the pathogen.

The term “antigen” as used herein refers to any protein, carbohydrate, or other moiety expressed by, or derived from, a pathogen that is capable of eliciting an immune response, preferably a protective immune response against the pathogen. The antigen may or may not be a structural component of the pathogen. Also contemplated to be within the term “antigen” are encoded antigens that can be translation products or polypeptides of various lengths. Antigens undergo normal host cell modifications such as glycosylation, myristoylation or phosphorylation. In addition, they can be designed to undergo intracellular, extracellular or cell-surface expression. Furthermore, they can be designed to undergo assembly and release from cells. The term “immunogen” may be used interchangeably with “antigen” to indicate a substance or molecule that elicits a specific immune response.

As used herein, the term “immunogenicity” means capable of producing an immune response in a host animal against an antigen or antigens. This immune response forms the basis of the protective immunity elicited by a vaccine against a specific infectious organism.

The term “immunizing” or “immunization”, as used herein, refers to the elicitation of an immune response in a patient that protects (partially or totally) from the manifestations of infection (i.e., disease) caused by a pathogen. A patient immunized by the present invention will not be infected by the pathogen or will be infected to a lesser extent than would occur without immunization. Immunizations may be either prophylactic or therapeutic in nature. That is, both previously uninfected and infected patients may be immunized with the present invention.

As used herein, the term “multivalent” means a vaccine composition comprising more than one immunogen, whether a nucleic acid construct, an antitoxin, an antiserum, or an immunogen derived from a bacteria, virus, parasite, fungi, or the like. Because a multivalent vaccine provides a greater

variety of antigens to the host's immune system, the immune response stimulated in the host is broader than that stimulated by only a single immunogen. The term "combination vaccine" may be used interchangeably with a "multivalent" vaccine.

5 As used herein, the term 'adjuvant" means a substance added to a vaccine composition to increase a vaccine's immunogenicity. The mechanism of how an adjuvant operates is not entirely known. Some adjuvants are believed to enhance the immune response by slowly releasing the antigen, while other adjuvants are strongly immunogenic in their own right and are believed to 10 function synergistically. Known vaccine adjuvants include, but are not limited to, oil and water emulsions (for example, complete Freund's adjuvant and incomplete Freund's adjuvant), Corynebacterium parvum, Bacillus Calmette Guerin, aluminum hydroxide, glucan, dextran sulfate, iron oxide, sodium alginate, Bacto-Adjuvant, certain synthetic polymers such as poly amino acids 15 and co-polymers of amino acids, saponin, "REGRESSIN" (Vetrepharm, Athens, Ga.), "AVRIDINE" (N, N-dioctadecyl-N',N'-bis(2-hydroxyethyl)-propanediamine), paraffin oil, and muramyl dipeptide. The term "adjuvant" as used in the present invention is also meant to encompass the use of liposomes, microspheres, and the like, as well as other methods known in the art to increase a vaccinate's 20 exposure to the vaccine and thereby elicit a stronger immune response in the vaccinate.

The term "adjuvant" also encompasses "genetic adjuvants," such as, but not limited to, immunomodulatory molecules encoded in a co-inoculated DNA or co-expressed in the same vaccine as a DNA immunogen. As used herein, the 25 term "genetic adjuvant" means an immunomodulatory or immunostimulatory molecule or fragment thereof including, but not limited to, interferon regulatory factors (IRFs) and apotosis-inducing caspase genes. IREs stimulate the activation of a number of inflammatory proteins that, in turn, help mobilize host immune responses. To date, nine cellular IRFs have been identified including 30 IRF-3 and IRF-7, which serve as direct transducers of virus mediated signaling pathways, and IRF-1, shown to be an activator of type I interferons. Other genetic adjuvants" contemplated to be within scope of the present invention include apotosis-inducing caspases. Caspases are cysteine proteases produced

as precursor molecules and activated by cleavage at aspartate residues. Proapoptotic caspases belong to one of two classes, those with long aminoterminal prodomains (class I caspases) and those with short or absent prodomains (class II caspases). In the caspase cascade that leads to apotosis, class I caspases are activated by oligomerization and, in turn, activate class II caspases that cleave the cellular proteins that result in apoptotic bodies.

As used herein, the term "pharmaceutically acceptable carrier" means a vehicle for containing the vaccine composition that can be injected into a patient without significant adverse effects. Suitable pharmaceutically acceptable carriers known in the art include, but are not limited to, sterile water, phosphate buffered saline, normal saline, glucose, dextrose, buffered solutions, and the like. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (i.e., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors, adjuvants, and the like.

The term "nucleic acid" as used herein refers to any natural and synthetic linear and sequential arrays of nucleotides and nucleosides, for example cDNA, genomic DNA, mRNA, tRNA, oligonucleotides, oligonucleosides and derivatives thereof. For ease of discussion, such nucleic acids may be collectively referred to herein as "constructs," "plasmids," or "vectors." Representative examples of the nucleic acids of the, present invention include bacterial plasmid vectors including expression, cloning, cosmid and transformation vectors such as, but not limited to, pBR322, animal viral vectors such as, but not limited to, modified adenovirus, influenza virus, polio virus, pox virus, retrovirus, and the like, vectors derived from bacteriophage nucleic acid, and synthetic oligonucleotides like chemically synthesized DNA or RNA. The term "nucleic acid" further includes modified or derivatised nucleotides and nucleosides such as, but not limited to, halogenated nucleotides such as, but not only, 5-bromouracil, and derivatised nucleotides such as biotin-labeled nucleotides.

The terms "polynucleotide", "oligonucleotide", and "nucleic acid sequence" are used interchangeably herein and include, but are not limited to, coding sequences (polynucleotide(s) or nucleic acid sequence(s) which are transcribed and translated into polypeptide *in vitro* or *in vivo* when placed under

the control of appropriate regulatory or control sequences), control sequences (e.g., translational start and stop codons, promoter sequences, ribosome binding sites, polyadenylation signals, transcription factor binding sites, transcription termination sequences, upstream and downstream regulatory domains, enhancers, silencers, and the like), and regulatory sequences (DNA sequences to which a transcription factor(s) binds and alters the activity of a gene's promoter either positively (induction) or negatively (repression)). No limitation as to length or synthetic origin are suggested by the terms described herein.

The term "isolated nucleic acid" as used herein refers to a nucleic acid with a structure (a) not identical to that of any naturally occurring nucleic acid or (b) not identical to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes, and includes DNA, RNA, or derivatives or variants thereof. The term includes, but is not limited to, the following: (a) a DNA which has the sequence of part of a naturally occurring genomic molecule but is not flanked by at least one of the coding sequences that flank that part of the molecule in the genome of the species in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic nucleic acid of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any vector or naturally occurring genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), ligase chain reaction (LCR) or chemical synthesis, or a restriction fragment; (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein, and (e) a recombinant nucleotide sequence that is part of a hybrid sequence that is not naturally occurring.

It is advantageous for some purposes that a nucleotide sequence is in purified form. The term "purified" in reference to nucleic acid represents that the sequence has increased purity relative to the natural environment.

As used herein the terms "polypeptide" and "protein" refer to a polymer of amino acids of three or more amino acids in a serial array, linked through peptide bonds. The term "polypeptide" includes proteins, protein fragments, protein analogues, oligopeptides and the like. The term "polypeptides"

contemplates polypeptides as defined above that are encoded by nucleic acids, produced through recombinant technology, isolated from an appropriate source, or are synthesized. The term "polypeptides" further contemplates polypeptides as defined above that include chemically modified amino acids or amino acids
5 covalently or noncovalently linked to labeling ligands.

The term "fragment" as used herein to refer to a nucleic acid (e.g., cDNA) refers to an isolated portion of the subject nucleic acid constructed artificially (e.g., by chemical synthesis) or by cleaving a natural product into multiple pieces, using restriction endonucleases or mechanical shearing, or a portion of
10 a nucleic acid synthesized by PCR, DNA polymerase, or any other polymerizing technique known in the art, or expressed in a host cell by recombinant nucleic acid technology known to one of skill in the art. The term "fragment" as used herein may also refer to an isolated portion of a polypeptide, wherein the portion of the polypeptide is cleaved from a naturally occurring polypeptide by
15 proteolytic cleavage by at least one protease, or is a portion of the naturally occurring polypeptide synthesized by chemical methods well known to one of skill in the art.

The term "gene" or "genes" as used herein refers to nucleic acid sequences (including both RNA or DNA) that encode genetic information for the
20 synthesis of a whole RNA, a whole protein, or any portion of such whole RNA or whole protein. Genes that are not naturally part of a particular organism's genome are referred to as "foreign genes", "heterologous genes" or "exogenous genes" while genes that are naturally a part of a particular organism's genome are referred to as "endogenous genes.

25 The term "expressed" or "expression" as used herein refers to the transcription from a gene to give an RNA nucleic acid molecule at least complementary in part to a region of one of the two nucleic acid strands of the gene. The term "expressed or expression" as used herein also refers to the translation from said RNA nucleic acid molecule to give a protein or polypeptide
30 or a portion thereof.

As used herein, the term "locus" or "loci" refers to the site of a gene on a chromosome. Pairs of genes control hereditary traits, each in the same position on a pair of chromosomes. These gene pairs, or alleles, may both be dominant

or both be recessive in expression of that trait. In either case, the individual is said to be homozygous for the trait controlled by that gene pair. If the gene pair (alleles) consists of one dominant and one recessive trait, the individual is heterozygous for the trait controlled by the gene pair. Natural variation in genes or nucleic acid molecules caused by, for example, recombination events or resulting from mutation, gives rise to allelic variants with similar, but not identical, nucleotide sequences. Such allelic variants typically encode proteins with similar activity to that of the protein encoded by the gene to which they are compared, because natural selection typically selects against variations that alter function. Allelic variants can also comprise alterations in the untranslated regions of the gene as, for example, in the 3' or 5' untranslated regions or can involve alternate splicing of a nascent transcript, resulting in alternative exons being positioned adjacently.

The term "transcription regulatory sequences" as used herein refers to nucleotide sequences that are associated with a gene nucleic acid sequence and which regulate the transcriptional expression of the gene. The "transcription regulatory sequences" may be isolated and incorporated into a vector nucleic acid to enable regulated transcription in appropriate cells of portions of the vector DNA. The "transcription regulatory sequence" may precede, but are not limited to, the region of a nucleic acid sequence that is in the region 5' of the end of a protein coding sequence that may be transcribed into mRNA. Transcriptional regulatory sequences may also be located within a protein-coding region, in regions of a gene that are identified as "intron" regions, or may be in regions of nucleic acid sequence that are in the region of nucleic acid.

The term "coding region" as used herein refers to a continuous linear arrangement of nucleotides that may be translated into a protein. A full length coding region is translated into a full length protein; that is, a complete protein as would be translated in its natural state absent any post-translational modifications. A full length coding region may also include any leader protein sequence or any other region of the protein that may be excised naturally from the translated protein.

The term "probe" as used herein, when referring to a nucleic acid, refers to a nucleotide sequence that can be used to hybridize with and thereby identify

the presence of a complementary sequence, or a complementary sequence differing from the probe sequence but not to a degree that prevents hybridization under the hybridization stringency conditions used. The probe may be modified with labels such as, but not only, radioactive groups, biotin, or any
5 other label that is well known in the art.

The term "nucleic acid vector" as used herein refers to a natural or synthetic single or double stranded plasmid or viral nucleic acid molecule that can be transfected or transformed into cells and replicate independently of, or within, the host cell genome. A circular double stranded plasmid can be
10 linearized by treatment with an appropriate restriction enzyme based on the nucleotide sequence of the plasmid vector. A nucleic acid can be inserted into a vector by cutting the vector with restriction enzymes and ligating the pieces together. The nucleic acid molecule can be RNA or DNA.

The term "expression vector" as used herein refers to a nucleic acid vector
15 that may further include at least one regulatory sequence operably linked to a nucleotide sequence coding for the desired protein. Regulatory sequences are well recognized in the art and may be selected to ensure good expression of the linked nucleotide sequence without undue experimentation by those skilled in the art. As used herein, the term "regulatory sequences" includes promoters,
20 enhancers, and other elements that may control expression. Useful promoters include constitutively expressed promoters as well as exogenously inducible promoters. Constitutively expressed promoters like the CMV early/intermediate promoter are expressed in most, if not all, tissues most of the time, in contrast to exogenously inducible promoters, which can be "turned on" in response to an
25 exogenously supplied agent or stimulus which is generally not an endogenous metabolite or cytokine. Examples include an antibiotic-inducible promoter, such as a tetracycline-inducible promoter, a heat-inducible promoter, a light-inducible promoter, or a laser inducible promoter (see, *inter ahia,* Halloran *et al.,* 2000; Gemer *et al.,* 2000; Rang and Will, 2000; Hagihara *et al.;* Huang *et al.,* 1999;
30 Forster. *et al.,* 1999; and Liu *et al.,* 1998)(the disclosures of which are herein incorporated by reference in their entireties).

Standard molecular biology textbooks such as Sambrook *et al.* eds., *"Molecular Cloning: A Laboratory Manual,"* 2nd ed., Cold Spring Harbor Press

(1989)(incorporated herein in its entirety by reference) may be consulted to design suitable expression vectors, promoters, and other expression control elements without undue effort. It should be recognized, however, that the choice of a suitable expression vector depends upon multiple factors including the choice of the host cell to be transformed and/or the type of protein to be expressed.

The terms "transformation" and "transfection" as used herein refer to the process of inserting a nucleic acid into a host. Many techniques are well known to those skilled in the art to facilitate transformation or transfection of a nucleic acid into a prokaryotic or eukaryotic organism. These methods involve a variety of techniques, such as treating the cells with high concentrations of salt such as, but not only a calcium or magnesium salt, an electric field, detergent, or liposome mediated transfection, to render the host cell competent for the uptake of the nucleic acid molecules.

The term "recombinant cell" refers to a cell that has a new combination of nucleic acid segments that are not covalently linked to each other in nature. A new combination of nucleic acid segments can be introduced into an organism using a wide array of nucleic acid manipulation techniques available to those skilled in the art. A recombinant cell can be a single eukaryotic cell, or a single prokaryotic cell, or a mammalian cell. The recombinant cell can harbor a vector that is extragenomic. An extragenomic nucleic acid vector does not insert into the cell's genome. A recombinant cell can further harbor a vector or a portion thereof that is intragenomic. The term intragenomic defines a nucleic acid construct incorporated within the recombinant cell's genome.

The term "recombinant nucleic acid" as used herein refers to combinations of at least two nucleic acid sequences that are not naturally found in a eukaryotic or prokaryotic cell. The nucleic acid sequences may include, but are not limited to nucleic acid vectors, gene expression regulatory elements, origins of replication, sequences that when expressed confer antibiotic resistance, and protein-encoding sequences. The term "recombinant polypeptide" is meant to include a polypeptide produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location, purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that normally observed in nature.

The term “DNA transcription unit” as used herein refers to a polynucleotide sequence that includes at least two components: antigen-encoding DNA and transcriptional promoter elements. A DNA transcription unit may optionally include additional sequences, such as enhancer elements, splicing signals, termination
5 and polyadenylation signals, viral replicons, and/or bacterial plasmid sequences. The DNA transcription unit can be produced by a number of known methods. For example, DNA encoding the desired antigen can be inserted into an expression vector to construct the DNA transcription unit, as described in Sambrook *et al.*. eds., *“Molecular Cloning: A Laboratory Manual,”* 2nd ed., Cold Spring Harbor
10 Press (1989)(incorporated herein in its entirety by reference).

The term “vaccine insert” as used herein refers to the DNA transcription unit of a pathogen. Preferably, the vaccine insert is a DNA transcription unit that can generate an immune response in a patient. For example, the vaccine insert of the present invention is contemplated to include at least one antigen derived
15 from a pathogenic virus, bacteria, parasite, fungi, or the like. Exemplary viruses include herpesvirus, orthomyxoviruses, rhinoviruses, picornaviruses, adenoviruses, paramyxoviruses, coronaviruses, rhabdoviruses, togaviruses, flaviviruses, bunyaviruses, rubella virus, reovirus, hepadna viruses, Ebola, retroviruses (including human immunodeficiency virus), and the like. Exemplary
20 bacteria include mycobateria, spirochetes, rickettsias, chlamydia, mycoplasma and the like. Exemplary parasites include protozoans like malaria, Cryptosproidium, *Leishmania, Giardia* and the like. Vaccine inserts can also be derived from trematode and cestode parasites including, but not limited to, tapeworms, hookworms, flukes, roundworms, and the like. Exemplary fungi include yeasts,
25 molds, and the like. One skilled in the art will appreciate that this list does not include all potential pathogens against which a protective immune response can be generated by the invention described herein.

The term “selectable marker gene” as used herein refers to an expressed gene that allows for the selection of a population of cells containing the selectable
30 marker gene from a population of cells not having the expressed selectable marker gene. For example, the “selectable marker gene” may be an antibiotic resistance gene” that can confer tolerance to a specific antibiotic by a microorganism that was previously adversely affected by the drug. Such

resistance may result from a mutation or the acquisition of resistance due to plasmids containing the resistance gene transforming the microorganism.

The term "terminator sequence" or "terminator" as used herein refers to nucleotide sequences that function to stop transcription. The terms "transcription" or "transcribe" as used herein refers to the process by which RNA molecules are formed upon DNA templates by complementary base pairing. This process is mediated by RNA polymerase.

The term "antibody" as used herein refers to polyclonal and monoclonal antibodies and fragments thereof, and immunologic binding equivalents thereof. The term "antibody" refers to a homogeneous molecular entity, or a mixture such as a polyclonal serum product made up of a plurality of different molecular entities, and may further comprise any modified or derivatised variant thereof that retains the ability to specifically bind an epitope. As used herein, the term epitope" refers to a part of the protein that can specifically bind to an antibody by fitting into the antigen-binding site of the antibody. A monoclonal antibody is capable of selectively binding to a target antigen or epitope.

The term "immunoglobulin polypeptide" as used herein refers to a polypeptide derived from a constituent polypeptide of an antibody. An "immunological polypeptide" may be, but is not limited to, an immunological heavy or light chain and may include a variable region, a diversity region, joining region and a constant region or any combination, variant or truncated form thereof. The term "immunological polypeptides" further includes single-chain antibodies comprised of, but not limited to, an immunoglobulin heavy chain variable region, an immunoglobulin light chain variable region and optionally a peptide linker.

The major immunological advantage of DNA-based vaccine compositions is the ability of the immunogen to be presented by both MHC class I and class II molecules. Endogenously synthesized proteins readily enter processing pathways for the loading of peptide epitopes onto MHC I as well as MHC II molecules. MHC I-presented epitopes raise cytotoxic T-cells (Tc) responses whereas MHC II-presented epitopes raise helper T-cells (Th). By contrast, immunogens that are not synthesized in cells are largely restricted to the loading of MHC II epitopes and the raising of Th but not Tc. When compared with live attenuated vaccines or recombinant viral vectors that produce immunogens in cells and raise both Th and

Tc, DNA vaccines have the advantages of not being infectious and of focusing the immune response on only those antigens desired for immunization. DNA vaccines also are advantageous because they can be manipulated relatively easily to raise type *I* or type 2 T-cell help. This allows a vaccine to be tailored for the type of immune response that will be mobilized to combat an infection. DNA vaccines are also cost effective because of the ease with which plasmids can be constructed using recombinant DNA technology, the ability to use a generic method for vaccine production (growth and purification of plasmid DNA), and the stability of DNA over a wide range of temperatures.

The best immune responses are achieved using highly active expression vectors modeled on those developed for the production of recombinant proteins (Robinson and Pertmer, 1998). The most frequently used transcriptional control elements include a strong promoter. One such promoter suitable for use in the present invention is the cytomegalovirus (CMV) early/intermediate promoter, although other promoters may be used in a DNA vaccine without departing from the scope the present invention. Other transcriptional control elements useful in the present invention include a strong polyadenylation signal such as, for example, that derived from a bovine growth hormone encoding gene, or a rabbit β globin polyadenylation signal (see, *inter alia,* Bohm *et al.,* 1996; Chapman *et al..,* 1991; Hartikka *et al.,* 1996; Manthorpe *et al.,* 1993; Montgomery *et al,* 1993). The CMV early/immediate promoter may be used with or without intron A (Chapman *et al.,* 1991). The presence of intron A increases the expression of many antigens from RNA viruses, bacteria, and parasites, presumably by providing the expressed RNA with sequences that support processing. It will be appreciated that expression also may be enhanced by other methods known in the art including, but not limited to, optimizing the codon usage of prokaryotic mRNAs for eukaryotic cells (Andre *et al..,* 1998; Uchijima *et al..,* 1998). Multicistronic vectors may be used to express more than one immunogen or an immunogen and a immunostimulatory protein (Iwasaki *et al..,* 1997a; Wild *et al..,* 1998).

Immunogens can also be engineered to be more or less effective for raising antibody or Tc by targeting the expressed antigen to specific cellular compartments. For example, antibody responses are raised more effectively by antigens that are displayed on the plasma membrane of cells, or secreted

therefrom, than by antigens that are localized to the interior of cells (Boyle *et al.*, 1997; Inchauspe *et al.*, 1997). Tc responses may be enhanced by using N-terminal ubiquitination signals, which target the DNA-encoded protein to the proteosome causing rapid cytoplasmic degradation and more efficient peptide
5 loading into the MHC I pathway (Rodriguez *et al.*, 1997; Tobery and Siliciano, 1997; Wu and Kipps, 1997). For a review on the mechanistic basis for DNA-raised immune responses, refer to Robinson and Pertmer (2000)(the disclosure of which is incorporated herein by reference in its entirety).

The effects of different conformational forms of proteins on antibody
10 responses, the ability of strings of MHC I epitopes (minigenes) to raise Tc responses, and the effect of fusing an antigen with immune-targeting proteins have been evaluated using defined inserts. Ordered structures such as virus-like particles appear to be more effective than unordered structures at raising antibody (Fomsgaard *et al.*, 1998). This is likely to reflect the regular array of an
15 immunogen being more effective than a monomer of an antigen at cross-linking Ig-receptors and signaling a B-cell to multiply and produce antibody. Recombinant DNA molecules encoding a string of MHC epitopes from different pathogens can elicit Tc responses to a number of pathogens (Hanke *et al.*, 1998b). These strings of Tc epitopes are most effective if they also include a Th epitope (Maecker *et al.*.
20 1998; Thomson *et al.*, 1998).

Another approach to manipulating immune responses in vaccinates is to fuse immunogens to immunotargeting or immunostimulatory molecules. To date, the most successful of these fusions have targeted secreted immunogens to antigen presenting cells (APC) or lymph nodes (Boyle *et al.*, 1997). Fusion of a
25 secreted form of human IgG with CTLA-4 increased antibody responses to the IgG greater than 1000-fold and changed the bias of the response from complement (C'-)dependent to C'-independent antibodies.

Fusions of human IgG with L-selectin also increase antibody responses but do not change the C'-binding characteristics of the raised antibody. Fusions
30 between antigens and cytokine cDNAs have resulted in more moderate increases in antibody, Th, and Tc responses (Hakim *et al.*, 1996; Maecker *et al.*, 1997). IL-4-fusions have increased antibody responses, whereas IL-12 and IL-1β have enhanced T-cell responses.

DNA delivery represents an important aspect of the present invention. Approaches to DNA delivery include, but are not limited to, injection of DNA in saline using a hypodermic needle or gene gun delivery of DNA-coated gold beads. Saline injections deliver DNA into extracellular spaces, whereas gene gun deliveries bombard DNA directly into cells. The saline injections require much larger amounts of DNA (100-1 000 times more) than the gene gun (Fynan *et al.*, 1993). These two types of delivery also differ in that saline injections bias responses in vaccinates towards type I T-cell help, whereas gene gun deliveries bias responses towards type 2 T-cell help (Feltquate *et al.*, 1997; Pertmer *et al.*, 1996). DNAs injected in saline rapidly spread throughout the body. DNAs delivered by the gun are more localized at the target site. Following either method of inoculation, extracellular plasmid DNA has a short half-life on the order of 10 minutes (Kawabata *et al.*, 1995; Lew *et al.*., 1995). Vaccination by saline injections can be intramuscular (i.m.) or intradermal (i.d.) (Fynan *et al.*, 1993).

Although intravenous and subcutaneous injections have met with different degrees of success for different plasmids (Bohm *et al.*, 1998; Fynan *et al.*, 1993), intraperitoneal injections have not met with success (Bohm *et al.*, 1998; Fynan *et al.* 1993). Gene gun deliveries can be administered to the skin or to surgically exposed muscle. Methods and routes of DNA delivery that are effective at raising immune responses in mice are effective in other species.

Immunization by mucosal delivery of DNA has been less successful than immunizations using parenteral routes of inoculation. Intranasal administration of DNA in saline has met with both good (Asakura *et al.*, 1997; Sasaki *et al.*, 1998b) and limited (Fynan *et al.*., 1993) success. The gene gun has successfully raised IgG following the delivery of DNA to the vaginal mucosa (Livingston *et al.*, 1995). Some success at delivering DNA to mucosal surfaces has also been achieved using liposomes (McCluskie *et al.*, 1998), microspheres (Chen *et al.*, 1998a; Jones *et al.*, 1997) and recombinant *Shigella* vectors (Sizemore et al., 1995; Sizemore et al., 1997).

The dose of DNA needed to raise a response depends upon the method of delivery, the host, the vector, the encoded antigen, and the adjuvant. The most profound effect is seen for the method of delivery. From 10 µg to l mg of DNA is generally used for saline injections of DNA, whereas from 0.2 µg to 20 µg of DNA

is used for gene gun deliveries of DNA. In general, lower doses of DNA are used in mice (10-100 μg for saline injections and 0.2 μg to 2 μg for gene gun deliveries), and higher doses in primates (100 μg to I mg for saline injections and 2 μg to 20 μg for gene gun deliveries). The much lower amount of DNA required for gene gun deliveries reflects the ability of the gold beads to directly deliver DNA into cells.

An example of the marked effect of an antigen on the immune response raised can be found in studies comparing the antibody responses raised in rabbits by DNAs expressing the influenza hemagglutinin (HA) or an immunodeficiency virus envelope glycoprotein (Env) (Richmond *et al.,* 1998). Under similar immunization conditions, the hemagglutinin-expressing DNA raised long lasting, high avidity, high titer antibody (~100 μg per ml of specific antibody), whereas the Env-expressing DNA raised only transient, low avidity, and low titer antibody responses (<10 μg per ml of specific antibody). The differences in raised antibody were hypothesized as resulting from the nature of the antigen administered: hemagglutinin is a T-dependent antigen, while the highly glycosylated immunodeficiency virus Env behaves as a T-independent antigen.

The present invention provides a novel vaccine useful for eliciting a protective response in a host against infection with an infectious agent such as the measles virus. The novel vaccine of the present invention comprises a DNA transcription unit of a pathogen such as a virus, bacteria, parasite or fungi as the immunogen and, optionally, a complement component to enhance antibody titers elicited in a vaccinate. For example, one embodiment of the present invention provides a DNA-based vaccine expressing a fusion of the measles hemagglutinin (H) protein and the complement component, C3d. However, other DNA immunogens such as influenza virus hemagglutinin, measles nucleoprotein genes, hemagglutinin genes from canine distemper virus, and the like are contemplated to be within the scope of the present invention.

An alternate embodiment of the present invention contemplates a vaccine comprising a plasmid expressing a secreted **(S)** form of H from measles virus fused to three tandem copies of the murine homologue of C3d (sH-3C3d). One expression vector useful in the present invention is pTR600; however, one of skill in the art will recognize that a number of vectors can be constructed to suitably express the vaccine insert of the present invention without undue experimentation.

One embodiment of the present invention comprises a plasmid vector having a vaccine insert comprising a secreted (s) form of H from measles virus fused to three tandem copies of the murine homologue of C3d (sH-3C3d). However, in view of the teachings herein, one skilled in the art will recognize that any vaccine insert known in the art can be used in the novel vaccine construct described herein including, but not limited to, viral pathogens like HIV, influenza, measles, herpes, Ebola, as well as inserts from other pathogenic bacteria, parasites, fungi, and the like. For example, the present invention contemplates inserts from influenza virus and modifications thereof, including influenza virus hemagglutinin (HA) or nucleoprotein (NP) genes, and influenza virus genes including all subtypes and modifications thereof. However, one skilled in the art will appreciate that the discussion about inserts derived from influenza virus genes are exemplary in nature and provided for the sake of illustration only.

The novel vaccine of the present invention comprises a DNA immunogen fused to at least one copy of a complement component, such as C3d (sH-3C3d). One consequence of complement activation in the human immune system is the covalent attachment of the C3d fragment of the third complement protein to the activating protein. C3d in turn binds to CD2l on B lymphocytes, a molecule with B cell stimulatory functions that amplify B lymphocyte activation (Fearon. D. T. and R. H. Carter, 1995). By directly stimulating antibody producing B cells through complement receptor 2 (CD2l), C3d enhances the height of antibody responses and expands the pool of anti-H specific B cells (Fearon, 1998). CD2l is complexed with the co-stimulator CD19 on B cells and to stimulate signaling pathways. The sH-3C3d fusion protein allows for a cross-linking of H-specific cell surface Ig and CD21, thereby leading to the expansion of B cells specific for H.

In prior studies in mice by Dempsey *et al.*, the fusion of two or three copies of C3d to a model antigen, hen egg lysozyme increased the efficiency of immunizations by more than 1000-fold (Dempsey *et al.*, 1996). Recently, the ability of a DNA vaccine, expressing a fusion of hemagglutinin (HA) from influenza virus or the gpl2O subunit of Env from HIV-1 and the C3d component of complement, to achieve an earlier and more efficient antibody response was examined by the present inventors (Ross *et al.*, 2000 and Ross *et al.*, 2001). Results demonstrated that mice vaccinated with DNA expressing a secreted

influenza HA fused to three copies of C3d (sHA-3C3d) generated antibody that underwent more rapid avidity maturation than antibody generated by secreted or transmembrane forms of HA. This resulted in more rapid appearance of hemagglutination inhibition (HI) activity and protective immunity (Ross *et al.*, 2000). DNA expressing HIV-1 Env gpl2O induced higher antibody responses to Env and a faster onset of avidity maturation than did the respective wild-type gp12O sequences in vaccinated mice (Ross *et al.*, 2001).

Also provided by the present invention is a vaccine composition useful for producing an immune response in a vaccinate that protects (partially or totally) from the manifestations of infection (i.e., disease) caused by a pathogen like measles. A vaccine composition according to the present invention comprises a viral DNA insert, like the secreted form of a measles hemagglutinin, fused to at least one copy of a complement component, like C3d, in a pharmaceutically acceptable carrier like phosphate buffered saline, sterile water, glucose, dextrose, and the like. The pharmaceutically acceptable carrier optionally comprises auxiliary substances such as adjuvants, both conventional and genetic; stabilizers like sugars and amino acids; mineral oil; vegetable oil; alum; aluminum phosphate; bentonite; silica; immune enhancers like thymosin and interleukins; polyactic acid; polyglycolic acid; wetting or emulsifying agents; pH buffering agents; gelling or viscosity enhancing additives; preservatives like thimerosal, gentamycin, or mixtures thereof; flavoring agents; colors; and the like, depending upon a variety of factors including, but not limited to, the final product desired, the route of administration, and the like.

Another aspect of the present invention involves the addition of an adjuvant to the composition of the present invention to increase immunogenicity of the resulting vaccine. A number of adjuvants known in the art are suitable for increasing the immunogenicity of a vaccine composition according to the present invention including, but not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, Cornebacterium parvum, Bacillus Calmette Guerin, aluminum hydroxide, glucan, dextran sulfate, DEAE dextran (diethyl amino ethylether dextran), iron oxide, sodium alginate, Bacto-Adjuvant, certain synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin, "REGRESSIN" (Vetrepharm, Athens, Ga.), "AVRIDINE" (N,N-dioctadecyl-N',N'-

bis(2-hydroxyethyl)propanediamine), paraffin oil, muramyl dipeptide and the like. The concentration of the adjuvant may be very broad depending upon a number of factors including the adjuvant used, the route of administration, and the final vaccine composition desired. Standard reference texts, such as VACCINE PROTOCOLS, edited by Robinson *et al*, Humana Press, Totowa, NJ, 1996, and REMINGTON'S PHARMACEUTICAL SCIENCE, **17**th edition, 1985 (the contents of which are herein incorporated by reference in their entirety) may be consulted to select a suitable adjuvant and a concentration range without undue experimentation. For example, aluminum hydroxide may be used in the range of up to about 1.5% depending on the route of administration. Concentrations in the range of 0.1 to 1.0% are more typical. Likewise the concentration of DEAE dextran may be broad, e.g., up to about 6% may be used. Oil-in-water adjuvants are typically used in the range of from about 0.01% to about 20%, but more typically is used in concentrations of less than 10%, e.g., from about 0.2 to about 5%.

The present invention also contemplates the use of genetic adjuvants, such as immunomodulatory molecules, co-expressed in the same vaccine construct as the vaccine immunogen or co-inoculated in a separate DNA vector. Examples of genetic adjuvants contemplated to be within scope of the present invention include, but are not limited to, interferon regulatory factors (IRFs), including IRF-1, IRF-3, IRF-7 and the like, and caspase genes, including mutant caspase genes and the like, that increase an immune response in a vaccine recipient without interfering with immunogen expression.

When the final vaccine composition comprises different adjuvanted antigens, whether the antigens comprise more than one DNA insert or a DNA insert combined with at least one conventional viral, bacterial, fungal or parasitic antigen, it is recognized that the change in the concentration of one adjuvant is likely to alter the preferred concentration of the other. Thus, the preferred combination and concentrations of adjuvants used will depend upon a number of considerations, including the antigen components used in the final vaccine and the human or animal patient contemplated for vaccination.

Additional agents useful in the vaccine composition of the present invention include agents to improve suspendability and dispersion in solution; a chelator like EDTA, a preservative like thimerosal or gentamycin, and the like. It is within the

abilities of those skilled in the art to select an appropriate vehicle for the DNA vaccine of the present invention taking into account a number of factors including, but not limited to the route of administration, the antigens contained within the vaccine composition and the age of the patient to be vaccinated.

5 Optionally, the vaccine composition may be rendered isotonic to decrease discomfort associated with administration by injection using any conventional technique as, for example, by dialysis against a salt solution that is isotonic with the blood of the vaccinate. Any salt solution suitable for use as an injection medium may be used to render the vaccine composition isotonic.

10 The present invention also contemplates treatment or modification of the vaccine composition depending upon the vaccine delivery method desired. For example, liposome encapsulation, emulsion, microencapsulation, microsphere preparation, the use of polylactic acid and polyglycolic acid, and the like is contemplated to be within the scope of the present invention.

15 In a similar fashion, the final vaccine composition may be subjected to freeze-drying or lyophilization using conventional methods to produce a dry formulation. In an alternate embodiment, the vaccine of the present invention may be used in its final state as a liquid by dividing the final composition into a vial of appropriate volume, for example from about 1 ml to about 50 ml, followed by 20 stopping and sealing the vial under sterile conditions.

The novel DNA insert of the present invention may also be used as one component in a vaccine composition comprising other bacterial, viral, parasitic or fungal antigens capable of inducing a protective immune response against a pathogen. For example, a vaccine composition comprising a secreted form of 25 measles hemagglutinin fused with at least one complement component and combined with other vaccine antigens, both conventional and DNA-derived, is contemplated to be within the scope of the present invention. For example, one embodiment of the present invention comprises a DNA vaccine insert comprising a secretory form of a measles Hemagglutinin fused with at least one copy of a 30 complement component like C3d combined with at least one other DNA vaccine antigen including, but not limited to, an influenza Hemagglutinin or nucleoprotein (NP).

A vaccine according to the present invention can be administered in a variety of ways to elicit an immune response in the recipient, including through any parenteral or topical route. For example, a recipient can be inoculated by intravenous, intraperitoneal, intradermal, subcutaneous or intramuscular methods. Inoculation can be, for example, with a hypodermic needle, needle-less delivery devices such as those that propel a stream of liquid into the target site, or with the use of a gene gun that bombards DNA on gold beads into the target site. A vector comprising the pathogen vaccine insert and the genetic adjuvant can be administered to a mucosal surface by a variety of methods including intranasal administration, i.e., nose drops or inhalants, or intrarectal or intravaginal administration by solutions, gels, foams, or suppositories. Alternatively, the vaccine vector can be orally administered in the form of a tablet, capsule, chewable tablet, syrup, emulsion, or the like. In an alternate embodiment, a dual-expression vaccine made according to the present invention can be administered transdermally, by passive skin patches, iontophoretic means, and the like.

A further aspect of the present invention provides a method for administering a DNA vaccine to a patient in a therapeutically effective amount to elicit a protective immune response against a pathogen, such as measles. The dosage of such a vaccine required to elicit a protective immune response will depend on a number of factors including the age, size, and health status of the patient to be vaccinated. Other factors like antigen concentration, additional vaccine components, and route of administration (i.e., subcutaneous, intradermal, oral, intramuscular or intravenous administration) will also impact the effective dosage. The route of administration chosen will also depend upon a number of factors including, but not limited to, the age and health status of the patient to be vaccinated vaccination history, the number and type of antigens included in the vaccine, and the like.

The dosage of vaccine to administer is easily determinable based on the antigen concentration of the vaccine, the route of administration, and the age and health status of the patient to be vaccinated. Each batch of vaccine antigen may be individually calibrated. Alternatively, methodical immunogenicity trials of different dosages, as well as LD5o studies and other screening procedures can be used to determine effective dosage for a vaccine composition of the present

invention without undue experimentation. From the examples presented below, it will be readily apparent what approximate dosage would be appropriate. The critical factor is that the dosage of the vaccine composition elicits at least a partial protective effect against natural infection, as evidenced by a reduction in the mortality and morbidity associated with natural infection.

Repeated vaccinations are usually preferable at periodic time intervals to enhance the immune response initially or after a long period of time since the last dose. In one embodiment of the present invention, the vaccine is administered as a parenteral injection (i.e., subcutaneously, intradermally, or intramuscularly, with intramuscular injection preferred in most cases) in repeated doses of from about one to about five doses, preferably about two to about four injections, and most preferable about two doses, which are given at intervals of about two to about six weeks, preferably from about two to about five weeks. However, one of skill in the art will recognize that the number of doses and the time interval between vaccinations depends on a number of factors including, but not limited to, the age of the patient to be vaccinated; the route of immunization; the amount of antigen available per dose; and the like. For initial vaccination, the period will generally be longer than a week and preferably will be between about two to about five weeks. For previously vaccinated patients, a booster vaccination at about an annual interval may be performed.

The present invention is further described with respect to the following examples. However, the scope of the invention is not intended to be, and should not be, construed to be limited thereby.

Example 1: Plasmid Constructions

As illustrated schematically in **Fig. 1**, a eukaryotic expression vector, pTR600, was constructed to contain two copies of the cytomegalovirus immediate-early promoter (CMV-IE) plus intron A (IA) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription (**Fig 1A**). The vector contains a multi-cloning site for the easy insertion of gene segments. Also, the vector contains the Col E1 origin of replication for prokaryotic replication and the Kanamycin resistance gene *(Kan')* for selection in antibiotic media **(Fig. 1A**). This vector

meets the guidelines for vector design as described in the December, 1996 *"Points to consider on plasmid DNA vaccines for preventive infectious disease indications"* document published by the U.S. Food and Drug Administration.

Vaccine Insert Hemagglutinin (H) cDNA sequences from the Edmonston strain of measles, as previously described by Yang *et at.* (1997), and C3d sequences were cloned and transferred into the pTR600 vaccine vector using unique restriction endonuclease sites **(Fig. 1B)**. The secreted version was generated by deleting the transmembrane and cytoplasmic domains of H (nucleotides 1-189 deleted). This was accomplished using PCR to clone a fragment of the H gene in frame at the alanine residue at amino acid position 64 with an N-terminal synthetic mimic of the tissue plasminogen activator (tpA) leader sequence (Torres *et al.,* 2000). - Vectors expressing sH-C3d fusion proteins were generated by cloning three tandem repeats of the mouse homologue of C3d in frame at the 3' end of the sH gene as previously described (Dempsey *et al..,* 1996; Ross *et al..,* 2000; and Ross *et al.,* 2001). The construct design was based upon that used in Dempsey *et at.* and used sequences from pSLG-C3d (Dempsey *et al..,* 1996). Linkers composed of two repeats of four glycines and a serine {$(G_4S)_2$} were fused at the junctures of H and C3d and between each C3d repeat. Potential proteolytic cleavage sites between the junctions of C3d and the junction of sH and C3d were mutated by using *Bam HI* and *Bgl II* fusion to mutate an Arg codon to a Gly codon (Dempsey *et al.,* 1996).

The plasmids were amplified in *Escherichia coli* strain,. DH5α, purified using anion-exchange resin columns (Qiagen, Valencia, CA) and stored at -20°C in dH_2O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260nm and 280nm.

Example 2:Transfections and Expression Analysis

A human embryonic kidney cell line, 293T, was transfected (5×10^5 cells/transfection) with 2 ~µg of DNA using 12% lipofectamine according to the manufacture's (Life Technologies, Grand Island, NY) guidelines. Supernatants were collected and stored at -20°C. Quantitative antigen capture ELISAs for H were conducted as previously described (Cardoso *et at.,* 1998).

Western hybridization analysis: 15 µgof supernatant or cell lysate was diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA) and loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and incubated with a 1:1000 dilution of polyclonal rabbit anti-HA antisera in PBS containing 0.1% Tween 20 and 1 % nonfat dry milk. After extensive washing, bound rabbit antibodies were detected using a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Results: Two expressing-expressing vaccine plasmids were constructed in the pTR600 vector, as described in Example 1 above, to express either a secreted form of H (sH) from the Edmonston strain of measles or a C3d-fusion of the secreted form of H (sH-3C3d). As illustrated in **Fig. 1**, sH represents the entire ectodomain of H, but excludes the transmembrane and cytoplasmic region. The cloning placed the N-terminal synthetic mimic of the tissue plasminongen activator (tPA) leader sequence in frame with the H sequence. As shown in **Fig. 1 B,** the tPA leader and H sequences were fused immediately 3' to the transmembrane domain of H. The sH-3C3d fusion protein was generated by cloning three tandem repeats of the mouse homologue of C3d in frame with the secreted H gene (Fig 1 b). The proteolytic cleavage sites, found at the junction between each C3d molecule as well as the junction between the H protein and the first C3d coding region, were destroyed by mutagenesis.

Fig. 2 shows the results of western blot analyses, revealing sH and sH-3C3d proteins of the expected sizes. Using a rabbit polyclonal antibody to MV H antisera, western blot analysis showed a broad band of ~7OkD corresponding to the secreted form of H in the supernatant of transfected cells. A higher molecular weight band at ~190 kD is consistent with the projected size of the sH-3C3d fusion protein. No evidence for the proteolytic cleavage of the sH-C3d fusion protein was seen by western analysis, nor was evidence observed of homologous recombination between the tandem repeats of murine C3d or homologous recombination between the two CMV promoter regions.

Human 293T cells were transiently transfected with 2 µg of plasmid and

both supernatants and cell lysates were assayed for H using an antigen capture ELISA. Results, summarized in Table I below, showed that the measles virus H was expressed at slightly lower levels by plasmids containing sH-3C3d as compared to those containing sH. Approximately 75% of the H protein was
5 secreted into the supernatant for both sH-DNA and sH-3C3d-DNA transfected cells.

Table 1. Measles H Expression in Transiently transfected 293T Cells

VACCINE	TOTAL*	LYSATE	SUPERNATENT**
sH	0.32 ± 0.05	0.07 ± 0.01	0.23 ± 0.02 (77 %)
SH-3C3d	0.18 ± 0.03	0.05 ± 0.01	0.13 ± 0.02 (72 %)

10 *Data are expressed as mean O.D. value of three independent experiments.
**Data are expressed as a per-cent of the total H antigen secreted into the supernatant as compared to the amount in the cell lysate fraction.

15 **Example 3: DNA immunization of Mice**

Six to eight week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were used for inoculations. Mice, housed in microisolator units and allowed free access to food and water, were cared for under USDA guidelines for laboratory animals. Mice were anesthetized with 0.03-0.04 ml of a mixture of 5ml
20 ketamine HCl (100mg/ml) and lml xylazine (20mg/ml). Gene gun immunizations were performed on shaved abdominal skin using the hand held Accell gene delivery system as described previously (Haynes *et al.*, 1994; Pertmer *et al.*, 1995; and Pertmer and Robinson, 1999). Mice were immunized with two gene gun doses containing 0.05 µg of DNA per 0.5mg of approximately 1µm gold beads (0.1
25 µg total DNA) (Bio-Rad, Hercules, CA) at a helium pressure setting of 400 psi. Controls were vaccinated with pTR600 vector only or, to control for the possible immunostimulatory sequences found in the linker region between gene segments, were vaccinated with vectors expressing a fusion of the tPA leader sequence and the three copies of murine C3d alone. At 4 and 26 weeks post vaccination, mice
30 were boosted with the same dose of DNA given in the first immunization.

Example 4: Antibody Response to Measles DNA Immunizations

To determine if a DNA vaccine expressing a fusion of measles H and the C3d component of complement could achieve earlier and more efficient anti-H antibody responses, BALB/c mice were vaccinated by DNA coated gold particles via gene gun with 0.1 µg dose inoculum as described in Example 3. At 4 and 26 weeks post vaccination, mice were boosted with the same dose of DNA given in the first immunization. A quantitative ELISA was performed to assess anti-H specific IgG levels was performed as previously described (Cardoso *et al.*, 1998). Briefly, Ltk cells constitutively expressing the H protein of MV (Beauverger *et al.*, 1993) were grown in 96-well plates. Antisera dilutions were incubated with the intact cells expressing H antigen. The anti-H antibodies were allowed to bind to the cells for 30 mm and the cells were fixed in 80% acetone. The specific antibody responses were detected with biotinylated anti-mouse IgG antibodies and the streptavidine-phosphatase alkaline system (Sigma). Antibody binding to Ltk cells not expressing H antigen was used to standardize the system. The results were calculated as the endpoint dilution titer.

Referring now to **Figure 3**, results show that by week 8 the anti-H antibody levels were higher in mice vaccinated with sH-3C3d-expressing DNA than in mice vaccinated with sH-expressing DNA, and these titers continued to rise until week 12. The anti-H titers from mice vaccinated with sH-3C3d DNA ranged from 800 to 3200 at weeks 12 and 16. However, after the third vaccination, anti-H titers in these same mice ranged from 7800 to 8600. In contrast, mice vaccinated with sH DNA had low to undetectable levels of anti-H antibody after the first two immunizations.

Controls vaccinated with pTR600 vector only had low to undetectable levels of antibody, while controls vaccinated with vectors expressing a fusion of the tPA leader sequence and the three copies of murine C3d elicited no anti-H antibodies and were similar to the levels seen with vector only (data not shown).

The titers peaked at 200 by week 6 in sera collected from mice vaccinated with sH-DNA and slowly declined until the third boost at week 26. At week 28, the titers in sera collected from mice vaccinated with sH-DNA ranged from 800 to 1600. After the third immunization, the anti-H antibodies titers from mice

vaccinated with sH-3C3d DNA were ~8 times higher than serum from mice vaccinated with sH DNA (**Fig. 3**).

Mice vaccinated using a DNA vaccine of the present invention, expressing a secreted form of H, did not have detectable levels of antibody until the third vaccination at week 28 post-prime. In contrast, mice vaccinated with DNA expressing the fusion of sH and 3C3d proteins elicited a faster onset of antibody (2 vaccinations), as well as higher levels of antibodies (**Fig. 3**). The increase in height of the antibody response to H was 7-15 fold higher in mice vaccinated with the C3d protein expressing constructs compared to mice vaccinated with DNA expressing sH only. The increase in antibody response with DNA expressing sH-3C3d is even more intriguing, since this plasmid expressed ~60% as much protein as plasmid expressing sH only (**Table 1**). This level of antibody increase with the C3d fusion construct was similar to previous studies in mice vaccinated with DNA expressing C3d fused to secreted versions of influenza expressing or HIV envelope gpl2O.

Example 5: Virus Neutralization Assays

Neutralization assays were conducted on Vero cells grown in 24-well plates (25). Briefly, 50 p.f.u. of the Edmonston strain of measles virus were mixed with serial dilution of sera, incubated for 1 h at 37°C and then inoculated onto plates. After 3 h, a 4% carboxymethyl cellulose overlay was added to each well and plates were incubated for 96 h, stained with 2% neutral red for 24 h, fixed with 10% formaldehyde and the plaques were counted. Neutralization titers are defined as the reciprocal dilution of sera required to reduce plaque formation by 50% or 90%. Preimmune sera served as negative controls.

Neutralization results: Examination of the serum for MV neutralization showed mice vaccinated with DNA expressing a secreted H-fused to three copies of C3d (sH-3C3d) generated a more rapid appearance and higher levels of neutralizing antibody activity than DNA expressing sH only. Virus neutralization titers up to 1700 were observed after the second inoculation of 0.1 µg of sH-3C3d expressing DNA. Neutralizing antibody studies detected higher titers of neutralizing activity against the prototype MV Edmonston strain in mouse sera elicited by the sH-3C3d constructs than in the sera of mice vaccinated with sH

expressing DNA. Mice vaccinated with sH-3C3d expressing plasmids had a sharp rise in neutralizing antibody levels that reached a plateau by week 12. In contrast, it took a third vaccination with sH expressing DNA to elicit detectable levels of neutralizing antibodies. Results are summarized as follows:

Table 2: Neutralizing Antibody Titers in Serum from Vaccinated Mice

WEEK	SH*	SH-3C3d*	SH**	SH-3C3d**
8	<100	250	<100	<100
12	<100	1700	<100	250
16	<100	1520	<100	<100
26	<100	400	<100	<100
28	250	750	<100	250

The neutralization assay was read as the endpoint dilution of serum that still inhibited plaque formation by the Measles virus (Edmonston strain) in Vero cells. *Neutralization titers were determined for 50% plaque reduction; ** Neutralization titers were determined for 90% plaque reduction

In addition to the increase in the overall antibody level, there was a faster onset of antibodies that could specifically neutralize MV in an *in vitro* infection assay (**Fig. 4**). After the second immunization, detectable levels of neutralizing antibodies were observed in mice vaccinated with DNA expressing sH-3C3d. The titer of the neutralizing antibody peaked at week 14 (1700 for 50% plaque reduction). In contrast, mice vaccinated with sH expressing DNA had low levels of neutralizing antibody even after the third vaccination (250 for 50% plaque reduction).

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The foregoing is illustrative of the present invention, and is not to be
10 construed as limiting thereof, the invention being defined by the following claims.

What is claimed is:

1. A vector comprising:

a selectable marker gene;

a prokaryotic origin of replication; and

5 a eukaryotic transcription cassette, said transcription cassette comprising a promoter sequence, a leader sequence, a nucleic acid encoding a fusion protein operatively associated with said promoter sequence, and a polyadenylation signal sequence;

said fusion protein comprising the measles hemagglutinin protein fused to
10 at least one complement component C3d.

2. The vector according to claim 1, wherein said measles hemagglutinin protein is a secreted form of the measles hemagglutinin protein.

15 3. The vector according to claim 1, said fusion protein comprising the measles hemagglutinin protein fused to three tandem repeats of the complement component C3d.

4. The vector according to claim 1, said expression cassette further
20 comprising a mutant caspase gene.

5. The vector according to claim 1, said expression cassette further comprising an interfereon regulatory factor.

25 6. The vector according to claim 1, wherein said vector is a DNA vector.

7. The vector according to claim 1, wherein said vector is a plasmid.

8. A pharmaceutical formulation comprising the vector according to claim 1
30 in a pharmaceutically acceptable carrier.

9. The pharmaceutical formulation according to claim 8, wherein said carrier is an aqueous carrier.

10. A pharmaceutical formulation consisting essentially of the vector according to claim 1 in lyophilized form.

5 11. A method of immunizing a patient against measles, comprising administering to said patient a nucleic acid vaccine that expresses a fusion protein, said fusion protein comprising the measles hemagglutinin protein fused to at least one complement component C3d in an amount effective to immunize said patient against measles.

10

12. The method according to claim 11, wherein said measles hemagglutinin protein is a secreted form of the measles hemagglutinin protein.

13. The method according to claim 11, said fusion protein comprising the 15 measles hemagglutinin protein fused to three tandem repeats of the complement component C3d.

14. The method according to claim 11, wherein said administering step is carried out by parenteral administration.

20

15. The method according to claim 11, wherein said administering step is carried out by topical administration.

16. The method according to claim 11, wherein said administering step is 25 carried out by gene gun/microprojectile bombardment.

17. The method according to claim 11, wherein from 10 micrograms to 1 milligram of said nucleic acid vaccine is delivered.

30 18. The method according to claim 11, wherein said patient is a human patient.

19. The method according to claim 11, wherein said nucleic acid vaccine is a DNA vaccine.

20. The method according to claim 11, wherein said nucleic acid vaccine is
5 a plasmid.

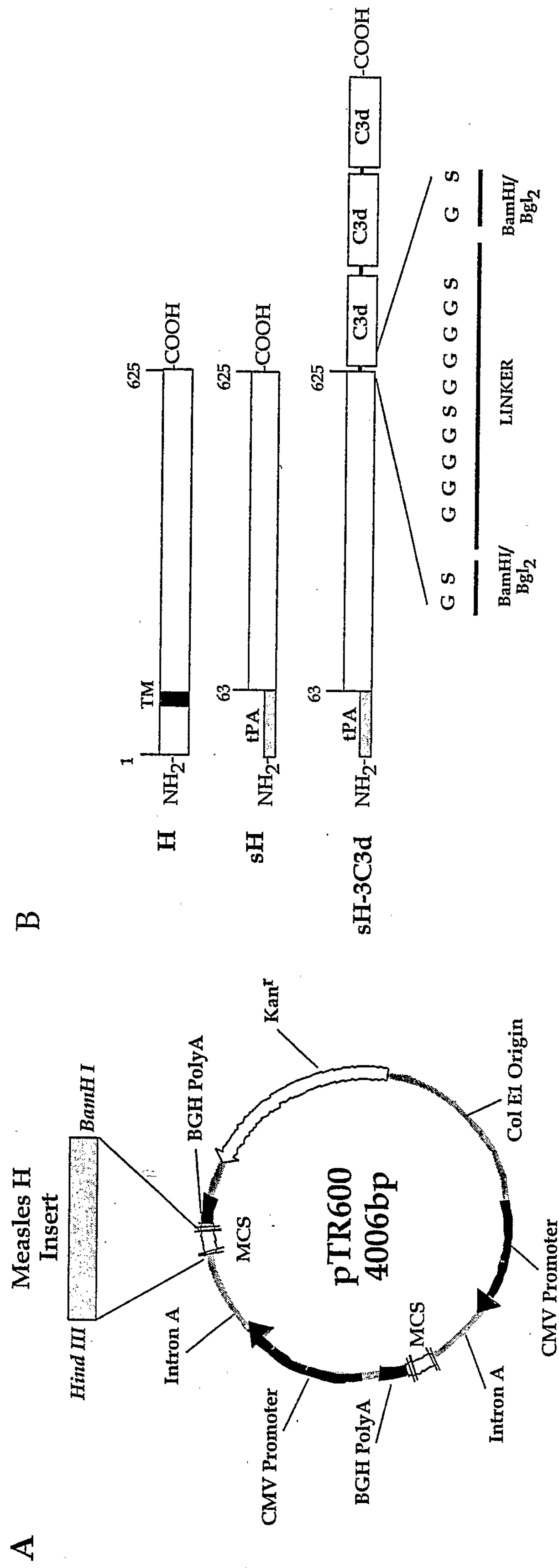

Figure 1

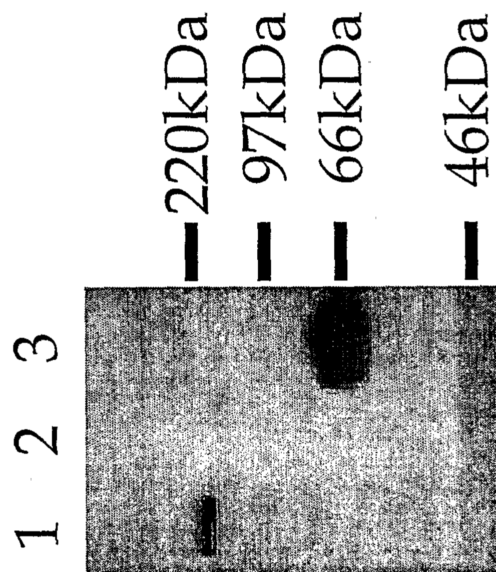

Figure 2

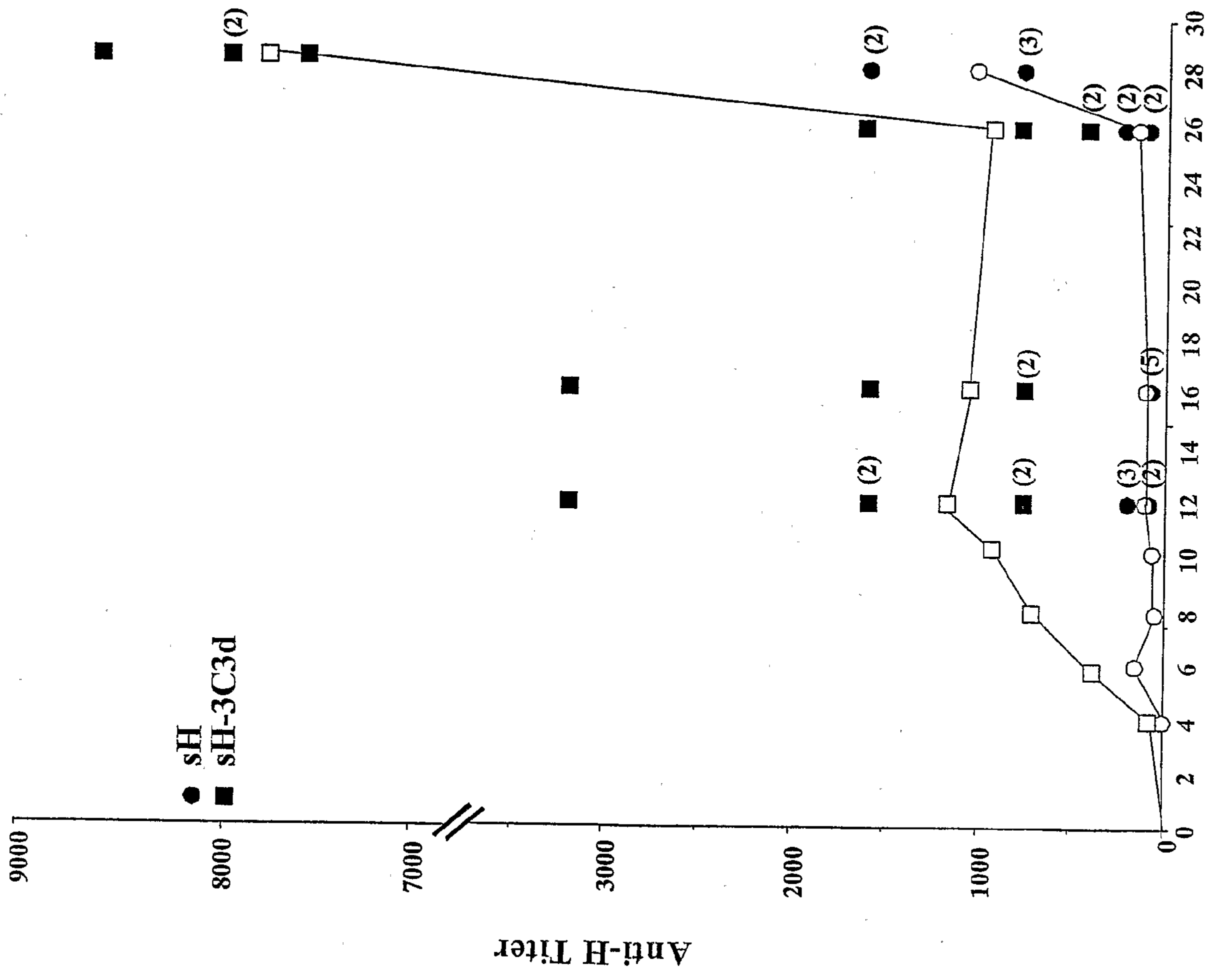

FIG 3