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(71) Demandeur/Applicant:
THE REGENTS OF THE UNIVERSITY OF MICHIGAN,
US
(72) Inventeurs/Inventors:
BAKER, JAMES R., JR., US;
BIELINSKA, ANNA, US;
MYC, ANDRZEJ, US
(74) Agent: SMART & BIGGAR

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(57) **Abrégé/Abstract:**

The present invention provides methods and compositions for the stimulation of immune responses. Specifically, the present invention provides methods and compositions for the use of nanoemulsion compounds as mucosal adjuvants to induce immunity against environmental pathogens. Accordingly, in some embodiments, the present invention provides nanoemulsion vaccines comprising a nanoemulsion and an inactivated pathogen or protein derived from the pathogen. The present invention thus provides improved vaccines against a variety of environmental and human-released pathogens.



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(71) Applicant (for all designated States except US): **THE REGENTS OF THE UNIVERSITY OF MICHIGAN** [US/US]; 3003 South State Street, Suite 2071, Ann Arbor, MI 48109 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BAKER, James, R., Jr.** [US/US]; 3997 Holden Drive, Ann Arbor, MI 48103 (US). **BIELINSKA, Anna** [PL/US]; 1652 Golden Lane, Ypsilanti, MI 48198 (US). **MYC, Andrzej** [US/US]; 5787 Glen Creek Court, Ann Arbor, MI 48108 (US).

(74) Agents: **SISK, Tyler, J.** et al.; Medlen & Carroll, LLP, 101 Howard Street, Suite 350, San Francisco, CA 94105 (US).

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(57) Abstract: The present invention provides methods and compositions for the stimulation of immune responses. Specifically, the present invention provides methods and compositions for the use of nanoemulsion compounds as mucosal adjuvants to induce immunity against environmental pathogens. Accordingly, in some embodiments, the present invention provides nanoemulsion vaccines comprising a nanoemulsion and an inactivated pathogen or protein derived from the pathogen. The present invention thus provides improved vaccines against a variety of environmental and human-released pathogens.



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NANOEMULSION VACCINES

This invention claims priority to U.S. Provisional Patent Application Serial Nos. 60/791,800, 60/791,759, and 60/791,758, each filed April 13, 2006, each of which is hereby incorporated by reference in its entirety.

This invention was made with government support under contract MDA 972-97-1-0007 awarded by the Defense Advanced Research Project Agency; and contract U54 AI57153-02 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention provides methods and compositions for the stimulation of immune responses. Specifically, the present invention provides methods of inducing an immune response to an agent (e.g., a bacteria or virus) in a subject (e.g., a human subject) and compositions useful in such methods (e.g., a nanoemulsion comprising a bacteria or virus or a component thereof). Compositions and methods of the present invention find use in, among other things, clinical (e.g. therapeutic and preventative medicine (e.g., vaccination)) and research applications.

BACKGROUND

Immunization is a principal feature for improving the health of people. Despite the availability of a variety of successful vaccines against many common illnesses, infectious diseases remain a leading cause of health problems and death. Significant problems inherent in existing vaccines include the need for repeated immunizations, and the ineffectiveness of the current vaccine delivery systems for a broad spectrum of diseases.

In order to develop vaccines against pathogens that have been recalcitrant to vaccine development, and/or to overcome the failings of commercially available vaccines due to expense, complexity, and underutilization, new methods of antigen presentation and immunization must be developed that allow for fewer immunizations, more efficient usage, and/or fewer side effects to the vaccine.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for the stimulation of immune responses. Specifically, the present invention provides methods of inducing an immune response to an agent (e.g., a bacteria or virus) in a subject (e.g., a human subject) and compositions useful in such methods (e.g., a nanoemulsion comprising a bacteria or virus or a component thereof). Compositions and methods of the present invention find use in, among other things, clinical (e.g. therapeutic and preventative medicine (e.g., vaccination)) and research applications.

Accordingly, in some embodiments, the present invention provides a method of inducing an immune response to an orthopox virus in a subject comprising: providing a composition comprising a nanoemulsion and an immunogen, wherein the immunogen comprises orthopox virus inactivated by the nanoemulsion; and administering the composition to the subject under conditions such that the subject generates an immune response to the orthopox virus. The present invention is not limited by the nature of the immune response generated. Indeed, a variety of immune responses may be generated and measured in a subject administered a composition comprising a nanoemulsion and an immunogen of the present invention including, but not limited to, activation, proliferation or differentiation of cells of the immune system (e.g., B cells, T cells, dendritic cells, antigen presenting cells (APCs), macrophages, natural killer (NK) cells, etc.); up-regulated or down-regulated expression of markers and cytokines; stimulation of IgA, IgM, or IgG titer; splenomegaly (e.g., increased spleen cellularity); hyperplasia, mixed cellular infiltrates in various organs, and other responses (e.g., of cells) of the immune system that can be assessed with respect to immune stimulation known in the art. In some embodiments, administering comprises contacting a mucosal surface of the subject with the composition. The present invention is not limited by the mucosal surface contacted. In some preferred embodiments, the mucosal surface comprises nasal mucosa. In some embodiments, administering comprises parenteral administration. The present invention is not limited by the route chosen for administration of a composition of the present invention. In some embodiments, inducing an immune response induces immunity to the orthopox virus in the subject. In some embodiments, the immunity comprises systemic immunity. In some embodiments, the immunity comprises mucosal immunity. In some embodiments, the immune response comprises increased expression of IFN- γ in the subject. In some embodiments, the immune response comprises a systemic IgG response to the inactivated

orthopox virus. In some embodiments, the immune response comprises a mucosal IgA response to the inactivated orthopox virus. The present invention is not limited by the type of orthopox virus used in a composition of the present invention. Indeed, a variety of orthopox viruses may be used including, but not limited to, variola virus, vaccinia virus, cowpox, monkeypox, gerbilpox, camelpox, among others. In some embodiments, the orthopox virus inactivated by the nanoemulsion is administered to the subject under conditions such that between 10 and 10^3 pfu of the inactivated virus is present in a dose administered to the subject. However, the present invention is not limited to this amount of orthopox virus administered. For example, in some embodiments, more than 10^3 pfu of the inactivated virus (e.g., 10^4 pfu, 10^5 pfu, or more) is present in a dose administered to the subject. In some embodiments, a 10% nanoemulsion solution is utilized to inactivate the orthopox virus. However, the present invention is not limited to this amount (e.g., percentage) of nanoemulsion used to inactivate a orthopox virus. For example, in some embodiments, a composition comprising less than 10% nanoemulsion is used for inactivation. In some embodiments, a composition comprising more than 10% nanoemulsion is used for inactivation. In some embodiments, the nanoemulsion comprises W₂₀5EC. The present invention is not limited by the type of nanoemulsion utilized. Indeed, a variety of nanoemulsions are contemplated to be useful in the present invention. For example, in some preferred embodiments, the nanoemulsion (e.g., for generating an immune response (e.g., for use as a vaccine)) comprises an oil-in-water emulsion, the oil-in-water emulsion comprising a discontinuous oil phase distributed in an aqueous phase, a first component comprising a solvent (e.g., an alcohol or glycerol), and a second component comprising a surfactant or a halogen-containing compound. The aqueous phase can comprise any type of aqueous phase including, but not limited to, water (e.g., dH_2O , distilled water, tap water) and solutions (e.g., phosphate buffered saline solution). The oil phase can comprise any type of oil including, but not limited to, plant oils (e.g., soybean oil, avocado oil, flaxseed oil, coconut oil, cottonseed oil, squalene oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, and sunflower oil), animal oils (e.g., fish oil), flavor oil, water insoluble vitamins, mineral oil, and motor oil. In some preferred embodiments, the oil phase comprises 30-90 vol% of the oil-in-water emulsion (i.e., constitutes 30-90% of the total volume of the final emulsion), more preferably 50-80%. While the present invention is not limited by the nature of the alcohol component, in some preferred embodiments, the alcohol is ethanol or methanol. Furthermore, while the present invention is not limited by

the nature of the surfactant, in some preferred embodiments, the surfactant is a polysorbate surfactant (e.g., TWEEN 20, TWEEN 40, TWEEN 60, and TWEEN 80), a phoxypolyethoxyethanol (e.g., TRITON X-100, X-301, X-165, X-102, and X-200, and TYLOXAPOL) or sodium dodecyl sulfate. Likewise, while the present invention is not limited by the nature of the halogen-containing compound, in some preferred embodiments, the halogen-containing compound comprises a cetylpyridinium halides, cetyltrimethylammonium halides, cetyldimethylethylammonium halides, cetyldimethylbenzylammonium halides, cetyltributylphosphonium halides, dodecyltrimethylammonium halides, tetradecyltrimethylammonium halides, cetylpyridinium chloride, cetyltrimethylammonium chloride, cetylbenzyltrimethylammonium chloride, cetylpyridinium bromide, cetyltrimethylammonium bromide, cetyldimethylethylammonium bromide, cetyltributylphosphonium bromide, dodecyltrimethylammonium bromide, or tetradecyltrimethylammonium bromide. Nanoemulsions of the present invention may further comprise third, fourth, fifth, etc. components. In some preferred embodiments, an additional component is a surfactant (e.g., a second surfactant), a germination enhancer, a phosphate based solvent (e.g., tributyl phosphate), a neutramingen, L-alanine, ammonium chloride, trypticase soy broth, yeast extract, L-ascorbic acid, lecithin, p-hydroxybenzoic acid methyl ester, sodium thiosulfate, sodium citrate, inosine, sodium hydroxide, dextrose, and polyethylene glycol (e.g., PEG 200, PEG 2000, etc.). In some embodiments, the oil-in-water emulsion comprises a quaternary ammonium compound. In some preferred embodiments, the oil-in-water emulsion has no detectable toxicity to plants or animals (e.g., to humans). In other preferred embodiments, the oil-in-water emulsion causes no detectable irritation to plants or animals (e.g., to humans). In some embodiments, the oil-in-water emulsion further comprises any of the components described above. Quaternary ammonium compounds include, but are not limited to, N-alkyldimethyl benzyl ammonium saccharinate, 1,3,5-Triazine-1,3,5(2H,4H,6H)-triethanol; 1-Decanaminium, N-decyl-N, N-dimethyl-, chloride (or) Didecyl dimethyl ammonium chloride; 2-(2-(p-(Diisobutyl)cresoxy)ethoxy)ethyl dimethyl benzyl ammonium chloride; 2-(2-(p-(Diisobutyl)phenoxy)ethoxy)ethyl dimethyl benzyl ammonium chloride; alkyl 1 or 3 benzyl-1-(2-hydroxyethyl)-2-imidazolinium chloride; alkyl bis(2-hydroxyethyl) benzyl ammonium chloride; alkyl dimethyl benzyl ammonium chloride; alkyl dimethyl 3,4-dichlorobenzyl ammonium chloride (100% C12); alkyl dimethyl 3,4-dichlorobenzyl ammonium chloride (50% C14, 40% C12, 10% C16); alkyl dimethyl 3,4-dichlorobenzyl ammonium chloride

(55% C14, 23% C12, 20% C16); alkyl dimethyl benzyl ammonium chloride; alkyl dimethyl benzyl ammonium chloride (100% C14); alkyl dimethyl benzyl ammonium chloride (100% C16); alkyl dimethyl benzyl ammonium chloride (41% C14, 28% C12); alkyl dimethyl benzyl ammonium chloride (47% C12, 18% C14); alkyl dimethyl benzyl ammonium chloride (55% C16, 20% C14); alkyl dimethyl benzyl ammonium chloride (58% C14, 28% C16); alkyl dimethyl benzyl ammonium chloride (60% C14, 25% C12); alkyl dimethyl benzyl ammonium chloride (61% C11, 23% C14); alkyl dimethyl benzyl ammonium chloride (61% C12, 23% C14); alkyl dimethyl benzyl ammonium chloride (65% C12, 25% C14); alkyl dimethyl benzyl ammonium chloride (67% C12, 24% C14); alkyl dimethyl benzyl ammonium chloride (67% C12, 25% C14); alkyl dimethyl benzyl ammonium chloride (90% C14, 5% C12); alkyl dimethyl benzyl ammonium chloride (93% C14, 4% C12); alkyl dimethyl benzyl ammonium chloride (95% C16, 5% C18); alkyl dimethyl benzyl ammonium chloride (and) didecyl dimethyl ammonium chloride; alkyl dimethyl benzyl ammonium chloride (as in fatty acids); alkyl dimethyl benzyl ammonium chloride (C12-C16); alkyl dimethyl benzyl ammonium chloride (C12-C18); alkyl dimethyl benzyl and dialkyl dimethyl ammonium chloride; alkyl dimethyl dimethylbenzyl ammonium chloride; alkyl dimethyl ethyl ammonium bromide (90% C14, 5% C16, 5% C12); alkyl dimethyl ethyl ammonium bromide (mixed alkyl and alkenyl groups as in the fatty acids of soybean oil); alkyl dimethyl ethylbenzyl ammonium chloride; alkyl dimethyl ethylbenzyl ammonium chloride (60% C14); alkyl dimethyl isopropylbenzyl ammonium chloride (50% C12, 30% C14, 17% C16, 3% C18); alkyl trimethyl ammonium chloride (58% C18, 40% C16, 1% C14, 1% C12); alkyl trimethyl ammonium chloride (90% C18, 10% C16); alkyl dimethyl(ethylbenzyl) ammonium chloride (C12-18); Di-(C8-10)-alkyl dimethyl ammonium chlorides; dialkyl dimethyl ammonium chloride; dialkyl dimethyl ammonium chloride; dialkyl dimethyl ammonium chloride; dialkyl methyl benzyl ammonium chloride; didecyl dimethyl ammonium chloride; diisodecyl dimethyl ammonium chloride; dioctyl dimethyl ammonium chloride; dodecyl bis (2-hydroxyethyl) octyl hydrogen ammonium chloride; dodecyl dimethyl benzyl ammonium chloride; dodecylcarbamoyl methyl dinethyl benzyl ammonium chloride; heptadecyl hydroxyethylimidazolinium chloride; hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine; myristalkonium chloride (and) Quat RNIUM 14; N,N-Dimethyl-2-hydroxypropylammonium chloride polymer; n-alkyl dimethyl benzyl ammonium chloride; n-alkyl dimethyl ethylbenzyl ammonium chloride; n-tetradecyl dimethyl benzyl ammonium chloride monohydrate; octyl decyl dimethyl ammonium

chloride; octyl dodecyl dimethyl ammonium chloride; octyphenoxyethoxyethyl dimethyl benzyl ammonium chloride; oxydiethylenebis (alkyl dimethyl ammonium chloride); quaternary ammonium compounds, dicoco alkyldimethyl, chloride; trimethoxysilyl propyl dimethyl octadecyl ammonium chloride; trimethoxysilyl quats, trimethyl dodecylbenzyl ammonium chloride; n-dodecyl dimethyl ethylbenzyl ammonium chloride; n-hexadecyl dimethyl benzyl ammonium chloride; n-tetradecyl dimethyl benzyl ammonium chloride; n-tetradecyl dimethyl ethylbenzyl ammonium chloride; and n-octadecyl dimethyl benzyl ammonium chloride. In some embodiments, the emulsion lacks any antimicrobial substances (i.e., the only antimicrobial composition is the emulsion itself). In some embodiments, the nanoemulsion is X8P. In some embodiments, the immunity protects the subject from displaying signs or symptoms of disease caused by an orthopox virus (e.g., vaccinia virus). In some embodiments, the immunity protects the subject from challenge with a subsequent exposure to live orthopox virus. In some embodiments, induction of an immune response protects a subject from morbidity and/or mortality associated with orthopox virus infection. In some embodiments, the composition further comprises an adjuvant. The present invention is not limited by the type of adjuvant utilized. A number of adjuvants that find use in the present invention are described herein. In some embodiments, the subject is a human.

The present invention also provides a composition for stimulating an immune response comprising a nanoemulsion and an orthopox virus inactivated by the nanoemulsion, wherein the composition is configured to induce immunity to the orthopox virus in a subject. In some embodiments, the nanoemulsion comprises W₂₀5EC. In some embodiments, the composition provides a subject between 10 and 10³ pfu of the inactivated virus when administered to the subject. In some embodiments, the composition provides a subject between 10³ and 10⁵ pfu of the inactivated virus when administered to the subject. In some embodiments, a dose of the composition that is administered to a subject comprises a 1% nanoemulsion solution. In some embodiments, the inactivated orthopox virus is heat stable in the nanoemulsion. In some embodiments, the orthopox virus is stable for greater than four weeks in the nanoemulsion. In some embodiments, the orthopox virus is vaccinia virus. The present invention is not limited by the type of orthopox virus used. Indeed, a variety of orthopox viruses can be used in a composition for stimulating an immune response including, but not limited to, variola virus, cowpox, monkeypox, gerbilpox, and camelpox. In some embodiments, the composition is diluted prior to administration to a

subject. In some embodiments, the subject is a human. In some embodiments, the immunity is systemic immunity. In some embodiments, the immunity is mucosal immunity. In some embodiments, the composition further comprises an adjuvant.

The present invention also provides a kit comprising a composition for stimulating an immune response comprising a nanoemulsion and an orthopox virus inactivated by the nanoemulsion, wherein the composition is configured to induce immunity to the orthopox virus in a subject, and instructions for administering the composition.

In some embodiments, the present invention provides a method of inducing an immune response to *B. anthracis* in a subject comprising providing a composition comprising a nanoemulsion and an immunogen, wherein the immunogen comprises a *B. anthracis* immunogen (e.g., recombinant protective antigen (rPA) of *B. anthracis*); and administering the composition to the subject under conditions such that the subject generates an immune response to *B. anthracis*. The present invention is not limited by the *B. anthracis* immunogen utilized. For example, in some embodiments, the immunogen is an isolated, purified or recombinant protein or peptide antigen, or derivative or variant thereof, selected from the group comprising, but not limited to, protective antigen (PA), lethal factor (LF), edema factor (EF), and PA degradation products. The present invention is not limited by the nature of the immune response generated. Indeed, a variety of immune responses may be generated and measured in a subject administered a composition comprising a nanoemulsion and an immunogen of the present invention including, but not limited to, activation, proliferation or differentiation of cells of the immune system (e.g., B cells, T cells, dendritic cells, antigen presenting cells (APCs), macrophages, natural killer (NK) cells, etc.); up-regulated or down-regulated expression of markers and cytokines; stimulation of IgA, IgM, or IgG titer; splenomegaly (e.g., increased spleen cellularity); hyperplasia, mixed cellular infiltrates in various organs, and other responses (e.g., of cells) of the immune system that can be assessed with respect to immune stimulation known in the art. In some embodiments, administering comprises contacting a mucosal surface of the subject with the composition. The present invention is not limited by the mucosal surface contacted. In some preferred embodiments, the mucosal surface comprises nasal mucosa. In some embodiments, administering comprises parenteral administration. The present invention is not limited by the route chosen for administration of a composition of the present invention. In some embodiments, inducing an immune response induces immunity to *B. anthracis* in the subject. In some embodiments, the immunity comprises systemic

immunity. In some embodiments, the immunity comprises mucosal immunity. In some embodiments, the immune response comprises increased expression of IFN- γ in the subject. In some embodiments, the immune response comprises a systemic IgG response. In some embodiments, the immune response comprises a mucosal IgA response. In some
; embodiments, the composition comprises between 1 and 300 μg of rPA. However, the present invention is not limited to this amount of recombinant protective antigen administered. For example, in some embodiments, more than 300 μg of rPA is present in a dose administered to the subject. In some embodiments, less than 1 μg of rPA is present in a dose administered to a subject. In some embodiments, the composition comprises a 10%
) nanoemulsion solution. However, the present invention is not limited to this amount (e.g., percentage) of nanoemulsion. For example, in some embodiments, a composition comprises less than 10% nanoemulsion. In some embodiments, a composition comprises more than 10% nanoemulsion. In some embodiments, a composition of the present invention comprises any of the nanoemulsions described herein. In some embodiments, the
; nanoemulsion comprises W₂₀5EC. The present invention is not limited by the type of nanoemulsion utilized.

In some embodiments, the immunity protects the subject from displaying signs or symptoms of disease caused by *B. anthracis*. In some embodiments, the immunity protects the subject from challenge with a subsequent exposure to live *B. anthracis*. In some
) embodiments, induction of an immune response protects a subject from morbidity and/or mortality associated with *B. anthracis* infection. In some embodiments, the composition further comprises an adjuvant. The present invention is not limited by the type of adjuvant utilized. In some embodiments, the adjuvant is a CpG oligonucleotide. A number of other adjuvants that find use in the present invention are described herein. In some embodiments,
; the subject is a human. In some preferred embodiments, immunity protects said subject from displaying signs or symptoms of anthrax.

The present invention also provides a composition for stimulating an immune response comprising a nanoemulsion and recombinant protective antigen of *B. anthracis*, wherein the composition is configured to induce immunity to *B. anthracis* in a subject. In
) some embodiments, the nanoemulsion comprises W₂₀5EC. In some embodiments, the composition provides the subject between 25 and 75 μg of the recombinant protective antigen when administered to the subject. In some embodiments, a dose of the composition administered to the subject comprises a 1% nanoemulsion solution. In some embodiments,

the recombinant protective antigen is heat stable in the nanoemulsion. In some embodiments, the recombinant protective antigen is stable for greater than four weeks in the nanoemulsion. In some embodiments, the composition is diluted prior to administration to a subject. In some embodiments, the subject is a human. In some embodiments, the immunity is systemic immunity. In some embodiments, the immunity is mucosal immunity. In some embodiments, the composition further comprises an adjuvant. In some embodiments, the adjuvant comprises a CpG oligonucleotide.

The present invention also provides a kit comprising a composition for stimulating an immune response comprising a nanoemulsion and recombinant protective antigen of *B. anthracis*, wherein the composition is configured to induce immunity to *B. anthracis* in a subject, and instructions for administering the composition. In some embodiments, the kit further comprises a device for administering the composition. The present invention is not limited by the type of device utilized for administering the composition. Indeed, a variety of devices are contemplated to be useful in a kit including, but not limited to, a nasal applicator, a syringe, a nasal inhaler and a nasal mister. In some embodiments, the kit comprises a composition comprising a nanoemulsion and a *B. anthracis* immunogen in contact with a device (e.g., a applicator). In some embodiments, the present invention provides systems and methods for large scale administration (e.g., to a population of a town, village, city, state or country) of a composition of the present invention (e.g., in response to an attack using a *Bacillus* pathogen).

In some embodiments, the present invention provides a method of inducing an immune response to HIV in a subject comprising providing a composition comprising a nanoemulsion and an immunogen, wherein the immunogen comprises recombinant gp120; and administering the composition to the subject under conditions such that the subject generates an immune response to the HIV. The present invention is not limited by the type of immunogen utilized (e.g., recombinant gp120). For example, in some embodiments, the immunogen is an isolated, purified or recombinant Tat, Nef or other immunogenic HIV protein, or derivative thereof. The present invention is not limited by the nature of the immune response generated. Indeed, a variety of immune responses may be generated and measured in a subject administered a composition comprising a nanoemulsion and an immunogen of the present invention including, but not limited to, activation, proliferation or differentiation of cells of the immune system (e.g., B cells, T cells, dendritic cells, antigen presenting cells (APCs), macrophages, natural killer (NK) cells, etc.); up-regulated or down-

regulated expression of markers and cytokines; stimulation of IgA, IgM, or IgG titer; splenomegaly (e.g., increased spleen cellularity); hyperplasia, mixed cellular infiltrates in various organs, and other responses (e.g., of cells) of the immune system that can be assessed with respect to immune stimulation known in the art. In some embodiments, administering comprises contacting a mucosal surface of the subject with the composition. The present invention is not limited by the mucosal surface contacted. In some preferred embodiments, the mucosal surface comprises nasal mucosa. In some embodiments, the mucosal surface comprises vaginal mucosa. In some embodiments, administering comprises parenteral administration. The present invention is not limited by the route chosen for administration of a composition of the present invention. In some embodiments, inducing an immune response induces immunity to said HIV in said subject. In some embodiments, the immunity comprises systemic immunity. In some embodiments, the immunity comprises mucosal immunity. In some embodiments, the immune response comprises increased expression of IFN- γ in the subject. In some embodiments, the immune response comprises a systemic IgG response. In some embodiments, the immune response comprises a mucosal IgA response. In some embodiments, the composition comprises between 15 and 75 μ g of recombinant gp120. However, the present invention is not limited to this amount of recombinant gp120 administered. For example, in some embodiments, more than 75 μ g of recombinant gp120 is present in a dose administered to the subject. In some embodiments, less than 15 μ g of recombinant gp120 is present in a dose administered to a subject. In some embodiments, the composition comprises a 10% nanoemulsion solution. However, the present invention is not limited to this amount (e.g., percentage) of nanoemulsion. For example, in some embodiments, a composition comprises less than 10% nanoemulsion. In some embodiments, a composition comprises more than 10% nanoemulsion. In some embodiments, the nanoemulsion comprises W₂₀5EC (See U.S. Pat. No. 6,015,832, hereby incorporated by reference in its entirety).. In some embodiments, the nanoemulsion comprises X8P (See U.S. Pat. No. 6,015,832, hereby incorporated by reference in its entirety).

The present invention also provides a kit comprising a composition for stimulating an immune response comprising a nanoemulsion and an HIV immunogen (e.g., recombinant gp120), wherein the composition is configured to induce immunity to HIV in a subject, and instructions for administering the composition. In some embodiments, the kit comprises a nanoemulsion in contact with an object (e.g., an applicator). In some embodiments, the kit

comprises a device for administering the composition. The present invention is not limited by the type of device included in the kit for administering the composition. Indeed, many different devices may be included in the kit including, but not limited to, a nasal applicator, a syringe, a nasal inhaler and a nasal mister. In some embodiments, the kit comprises a vaginal applicator, vaginal mister or other type of device for vaginal administration (e.g., to the vaginal mucosa) of a composition of the present invention. In some embodiments, a kit comprises a birth control device (e.g., a condom, an IUD, sponge, etc.) coated with a nanoemulsion composition of the present invention. In some embodiments, a nanoemulsion composition of the present invention is mixed in a douche or a suppository or a lubricant (e.g., sexual lubricant). In some embodiments, the present invention provides systems and methods (e.g., using a nanoemulsion composition of the present invention) for large scale administration (e.g., to a population of a city, village, town, state or country). In preferred embodiments, such large scale administrations are carried out in a manner that is easy to use (e.g., nasal administration) and that is culturally sensitive (e.g., so as not to offend those being administered a composition of the present invention).

DESCRIPTION OF THE FIGURES

The following figures form part of the present specification and are included to further demonstrate certain aspects and embodiments of the present invention. The invention may be better understood by reference to one or more of these figures in combination with the description of specific embodiments presented herein.

Figure 1 shows complete virus inactivation with nanoemulsion. (A) Plaque reduction assay (PRA) of VV_{WR}. (B) Luciferase assay of VV_{WR-Luc}. Luciferase activity is presented in relative light units (RLU). (C) PCR analysis of lung DNA. Lane 1: DNA size marker; lane 2: primers, no DNA; lane 3: no Taq; lane 4: 10⁵/Fk lung DNA; lanes 5-7: 10⁵/Fk/NE lung DNA; lanes 8-10: 10⁵/NE lung DNA; lane 11: control - VV DNA mixed with lung DNA. Arrows indicate amplified viral template and primers. (D) *In vivo* bioluminescence imaging of mice after intranasal infection with live VV_{WR-Luc} and with 10⁵ pfu of NE-killed virus. Circles visible in some images indicate region-of-interest (ROI) for photon flux analysis.

Figure 2 shows immunogenicity of mucosal nanoemulsion vaccine in mice. (A) Development of serum anti-VV IgG antibody response in mice vaccinated with various formulations of killed virus vaccine: 10⁵/NE (filled circle), 10³/NE (open circle), 10⁵/Fk/NE

(filled triangle), 10^3 /Fk/NE (open triangle), 10^5 /Fk (filled diamond) and 10^3 /Fk (open diamond). Arrows indicate i.n administrations of the vaccine. Insert: Comparison of serum anti-VV IgG after one or three vaccinations with 10^5 /NE vaccine. Data presented as mean of the individual anti-VV IgG concentrations $\pm$ sem. (B) Secretory anti-VV IgA antibody in BAL. Results are presented as mean concentrations ($\pm$ sem) of IgA obtained in assays performed with individual and pooled BAL fluids.

Figure 3 shows virus neutralizing antibodies. Assays were performed with both individual and with pooled sera obtained after one, two and three vaccinations. Insert: Detection of virus neutralizing activity in BAL. Assays were performed with individual and pooled BAL fluids collected at the conclusion of the experiment at 16 weeks. Results were normalized and presented as NT₅₀ of the viral PRA.

Figure 4 shows vaccinia-specific cellular immune responses. The INF- γ expression *in vitro* in splenocytes stimulated with 10^3 and 10^4 pfu of live VV_{WR}. The data show a specific INF- γ response to the virus in splenocytes from animals immunized with vaccinia virus inactivated by NE.

Figure 5 shows intranasal challenge with live vaccine virus. (A) Survival curves for mice vaccinated with 10^5 pfu of killed VV_{WR} in various vaccine formulations: VV/NE, VV/Fk/NE and VV/Fk, after i.n. challenge with $10\times$ LD₅₀ VV_{WR-Luc}. (B) Bioluminescence images of representative vaccinated (upper panel) and control mouse (lower panel). Images were recorded 2 to 5 days after challenge.

Figure 6 shows the stability of vaccine preparation. (A) 0.5 μ g of rPA protein was incubated 24 hours in saline and 1% NE at room temperature and analyzed using non-reducing 10% PAGE. Silver staining demonstrated low molecular weight fragments after incubation of the antigen without nanoemulsion (saline). (B) Micrographs of the NE and rPA/NE mix show no alteration in the emulsion after mixing with antigen (400 $\times$ magnification).

Figure 7 shows effect of nanoemulsion on the cellular uptake of rPA protein. Jaws II dendritic cells were incubated with either (A) medium, (B) 0.1 μ g/ml of PA-FITC alone, (C) 0.1 μ g/ml of PA-FITC mixed with 0.001% NE or (D) 1 μ g/ml of PA-FITC mixed with 0.001% NE. Green fluorescence indicates that the rPA was effectively internalized only when administered with nanoemulsion.

Figure 8 shows time course of the serum anti-PA IgG in mice. Mice were intranasally immunized with two doses of vaccine (arrows). (A) Induction of the anti-PA

IgG in CBA/J mice vaccinated with 20 μ g rPA and increasing concentration of NE. (A, insert). The anti-PA IgG subtypes in CBA/J mice immunized with rPA/NE. Data are presented as ratios of individual IgG2a, IgG2b and IgG3 titers versus IgG1 titer. (B) Anti-PA IgG in Balb/c mice vaccinated with various formulations of rPA vaccine. The results are presented as the mean $\pm$ sem of individual serum anti-PA IgG endpoint titers. (*) Indicates statistical difference between the titer achieved with rPA/NE vaccination and the antibody titers in the other groups ($p < 0.05$). (B, insert) Documentation of anti-PA IgE in mice immunized with rPA/Alu. Immuno-dot-blot of 1:10 to 1:80 dilutions of pooled sera from mice immunized with rPA/NE (1), rPA/MPL A (2), rPA/CpG (3), control (4) and rPA/Alu (5).

Figure 9 shows anti-PA IgA and IgG antibodies in bronchial lavage. The anti-PA IgA (A) and anti-PA IgG (B) determined by ELISA of bronchial alveolar lavage (BAL) from Balb/c mice vaccinated with various formulations of vaccine. Anti-PA IgA and anti-PA IgG antibodies are expressed as the mean $\pm$ sem of antibody concentrations.

Figure 10 shows lethal toxin (LeTx) neutralization in vitro. RAW264.7 cells were treated with the anthrax LeTx that had been preincubated with a serial dilution of immune, pooled Balb/c sera. Bars represent the antibody dilution in which cells retain 50% viability (NC₅₀).

Figure 11 shows PA-specific induction of splenocyte proliferation in vitro. Splenocytes isolated from immunized mice were stimulated with rPA (5 μ g/ml) for 72 hours. Proliferation indexes were calculated as a ratio of the activity in rPA-stimulated cells to the activity in resting splenocytes. (*) Indicates statistical difference between groups ($p < 0.05$).

Figure 12 shows immune response and survival of guinea pigs intranasally immunized with rPA/NE vaccine. Hartley guinea pigs were vaccinated with 2 doses of vaccine (day 1 and at 4 weeks as documented by arrows). (A) Anti-PA IgG in guinea pig serum. Antibody titers were determined at 3- to 4-week intervals with serum anti-PA IgG measured by ELISA (mean endpoint titers $\pm$ sem). (B) Intradermal challenge. At 6 months, guinea pigs were i.d injected with 1000 $\times$ LD₅₀ of Ames spores and mortality was monitored for 14 days. For vaccinated and control animals (B, insert) LeTx neutralization was performed at 22 weeks before the challenge. The antibody titer in which RAW264 cells retained 50% viability (NC₅₀) is determined from the cell viability obtained in at least two assays each performed in triplicate. (*) Indicates statistically significant difference as

compared to unvaccinated animals ($p < 0.001$).

Figure 13 shows immune response and intranasal challenge of guinea pigs intranasally vaccinated with rPA/NE vaccine. Hartley guinea pigs vaccinated on day 1 and at 4 weeks. (A) Anti-PA IgG and LeTx neutralizing antibody titers in serum. Antibody titers were determined at 3 and 6 weeks and are presented as the mean $\pm$ sem of individual serum anti-PA IgG endpoint titers. The LeTx neutralization assay cell was performed before the challenge, with values representing mean titers in which RAW264 cells retained 50% viability (NC_{50}). (B and C) Survival Curves after Intranasal Challenge. At 7 weeks guinea pigs were infected with i.n. instillation of 10 LD_{50} (B) and $100 \times LD_{50}$ (C) of Ames spores, and animals were monitored up to 16 days. (*) Indicates $p < 0.05$ between all vaccinated groups as compared to unvaccinated animals.

Figure 14 shows antibody response in mice intranasally vaccinated with two serotypes of recombinant gp120 and nanoemulsion adjuvant. (A) Induction of serum anti-gp120_{BaL} IgG in mice immunized with gp120_{BaL} mixed with 0.1%, 0.5% and 1% NE. Anti-gp120_{BaL} IgG antibodies were measured at 6 weeks (after two doses) and 12 weeks (after three doses). Intranasal (i.n.) and intramuscular (i.m.) routes of immunization are indicated in the Figure. (B) Induction of anti-gp120_{SF162} IgG in mice i.n. immunized with two doses of gp120_{SF162} in 1% NE alone or with addition of CpG or MPL A. Anti-gp120 IgG antibodies were measured at 6 weeks. Anti gp120 antibody levels are presented as a mean of endpoint titers ($\pm$ s.d.) in serum of individual animals. Cross-reactivity of the anti-gp120 antibodies. Serum IgG from mice immunized with either (C) gp120_{BaL} or (D) gp120_{SF162} reacts both with an autologous and with heterologous serotypes of antigen. Data presented as titration curves of pooled anti-gp120 sera on plates coated with either gp120_{SF162} or 120_{BaL} serotypes of antigen.

Figure 15 shows nasal immunization with gp120/NE induces mucosal IgA. (A) Secretory anti-gp120 IgA in bronchial lavage (BAL), and (B) in serum and in the vaginal washes of mice vaccinated with gp120_{BaL} and NE adjuvant. Anti-gp120 IgA concentration is presented as mean absorbance (OD 405 nm $\pm$ s.d.) obtained in ELISA performed with 1:2 diluted BAL fluids (A), undiluted vaginal washes, and 1:50 diluted serum (B). Statistically significant differences were observed between gp120/saline and each gp120/NE groups ($p < 0.05$).

Figure 16 shows (A) Antigen-specific splenocyte proliferation. Splenocytes from immunized animals were stimulated *in vitro* with 2 μ g/ml of autologous recombinant

gp120_{BaL}. Cell proliferation was normalized to controls and presented as mean $\pm$ s.d. of individual proliferation indexes. The differences between the gp120_{BaL}/saline and the gp120_{BaL}/NE groups were statistically significant ($p < 0.05$). (B) Antigen-specific activation of cytokine production in splenocytes *in vitro*. Splenocytes from immunized mice were activated with 2 μ g/ml of autologous and heterologous serotypes of gp120 (BaL and SF162, respectively) and with 20 μ M of the V3 loop peptide. The released IFN- γ was determined by ELISA and concentration is presented as a mean of individual samples $\pm$ s.d.

Figure 17 shows (A) Nasal immunization of guinea pig. Hartly guinea pigs (GP) were vaccinated in the prime-boost schedule with 50 μ g gp120_{SF162} in 1% NE. The serum IgG antibody response toward gp120_{SF162} and BaL serotypes was measured at six weeks. Anti-gp120 IgG are presented as absorption values (OD 405 nm, $\pm$ s.d.) obtained in ELISA with 1:200 dilution of serum using plates coated with gp120_{SF162}gp (autologous) and 120_{BaL} (heterologous) serotypes of antigen. (B) Neutralizing antibody produced by i.n. immunization with gp120_{SF162}/NE. The neutralization of laboratory strains and primary isolates of HIV were performed in the TZM-BL cell system. NT₅₀ values represent the serum dilution at which relative luminescence units (RLU) were reduced 50% compared to virus control. Individual preimmune sera were used to evaluate nonspecific antiviral activity.

GENERAL DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for the stimulation of immune responses. Specifically, the present invention provides methods of inducing an immune response against a pathogen (e.g., vaccinia virus, *Bacillus anthracis*, HIV, etc.) in a subject (e.g., a human subject) and compositions useful in such methods (e.g., a nanoemulsion comprising a pathogen inactivated by the nanoemulsion, or an immunogenic portion thereof). Compositions and methods of the present invention find use in, among other things, clinical (e.g. therapeutic and preventative medicine (e.g., vaccination)) and research applications. In some embodiments, the pathogen is mixed with the nanoemulsion prior to administration for a time period sufficient to inactivate the pathogen. In others, protein components (e.g., isolated or purified protein, or recombinant protein) from a pathogen are mixed with the nanoemulsion.

Although an understanding of the mechanism is not necessary to practice the present

invention and the present invention is not limited to any particular mechanism of action, in some embodiments, NE treatment (e.g., neutralization of a pathogen with a NE of the present invention) preserves important antigenic epitopes (e.g., recognizable by a subject's immune system), stabilizing their hydrophobic and hydrophilic components in the oil and water interface of the emulsion (e.g., thereby providing one or more immunogens (e.g., stabilized antigens) against which a subject can mount an immune response). In other embodiments, because NE formulations penetrate the mucosa through pores, they carry immunogens to the submucosal location of dendritic cells (e.g., thereby initiating and/or stimulating an immune response). Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, combining a NE and an immunogenic protein (e.g., rPA from *B. anthracis*, or gp120 from HIV, etc.) stabilizes the immunogen and provides a proper immunogenic material for generation of an immune response.

Dendritic cells avidly phagocytose NE oil droplets and this could provide a means to internalize immunogens (e.g., antigenic proteins or peptide fragments thereof) for antigen presentation. While other vaccines rely on inflammatory toxins or other immune stimuli for adjuvant activity (See, e.g., Holmgren and Czerkinsky, *Nature Med.* 2005, 11; 45-53), NEs have not been shown to be inflammatory when placed on the skin or mucous membranes in studies on animals and in humans. Thus, although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, a composition comprising a NE of the present invention (e.g., a composition comprising NE and an immunogen (e.g., a NE inactivated pathogen (e.g., a virus (e.g., VV))) may act as a "physical" adjuvant (e.g., that transports and/or presents immunogens (e.g., Vaccinia proteins) to the immune system. In some preferred embodiments, mucosal administration of a composition of the present invention generates mucosal (e.g., signs of mucosal immunity (e.g., generation of IgA antibody titers) as well as systemic immunity.

Both cellular and humoral immunity play a role in protection against multiple pathogens and both can be induced with a NE composition (e.g., comprising a pathogen inactivated by the nanoemulsion, or an immunogenic portion of a pathogen) of the present invention. For example, vaccinia-specific antibody titers are considered important for protective immunity in human subjects and in animal models of vaccination (See, e.g.,

Hammarlund et al, Nat. Med. 2003, 9; 1131-1137). Several studies have identified proteins important for the elicitation of neutralizing antibodies (See, e.g., Galmiche et al, Virology, 1999, 254; 71-80; Hooper et al, Virology, 2003, 306; 181-195). A recent trial of dilutions of the licensed smallpox vaccine (Dryvax) in human volunteers, confirmed that pustule formation strongly correlated with development of both specific antibodies and induction of cytotoxic T lymphocytes (CTL) and elevated INF- γ T cell responses (See, e.g., Greenberg et al, 2005, 365; 398-409). Induction of IFN- γ is suggestive of activation of specific MHC class I-restricted CD8+ T cells. These types of cells have been implicated in the recognition and clearance of Vaccinia infected cells, and for maintenance of immunity after vaccination (See, e.g., Earl et al, Nature, 2004; 482; 182-185; Hammarlund et al, Nat. Med. 2003, 9; 1131-1137; Edghill-Smith et al, Nature Med. 2005, 11; 740-747).

Thus, in some embodiments, administration (e.g., mucosal administration) of a composition of the present invention (e.g., NE-killed orthopox virus (e.g., VV)) to a subject results in the induction of both humoral (e.g., development of specific antibodies) and cellular (e.g., cytotoxic T lymphocyte) immune responses (e.g., against the orthopox virus). In some preferred embodiments, a composition of the present invention (e.g., NE-killed orthopox virus (e.g., VV) or a NE and one or more immunogens) is used as a vaccine (e.g., a smallpox vaccine, an anthrax vaccine, an influenza vaccine, etc.).

Furthermore, in some embodiments, a composition of the present invention (e.g., a composition comprising a NE and an immunogen) induces (e.g., when administered to a subject) both systemic and mucosal immunity. Thus, in some preferred embodiments, administration of a composition of the present invention to a subject results in protection against an exposure (e.g., a lethal mucosal exposure) to a pathogen (e.g., a virus (e.g., an orthopox virus (e.g., VV))). Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, mucosal administration (e.g., vaccination) provides protection against pathogen infection (e.g., that initiates at a mucosal surface). Although it has heretofore proven difficult to stimulate secretory IgA responses and protection against pathogens that invade at mucosal surfaces (See, e.g., Mestecky et al, Mucosal Immunology. 3rd edn. (Academic Press, San Diego, 2005)), the present invention provides compositions and methods for stimulating mucosal immunity (e.g., a protective IgA response) from a pathogen in a subject.

In some embodiments, the present invention provides a composition (e.g.,

comprising a NE and an immunogen) to serve as a mucosal vaccine. This material can easily be produced from purified virus and/or bacteria and/or protein or recombinant protein and induces both mucosal and systemic immunity. The ability to produce this formulation rapidly and administer it via mucosal instillation provides a vaccine that can be used for general vaccination needs as well as in large-scale outbreaks or emergent situations.

DEFINITIONS

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

As used herein, the term "microorganism" refers to any species or type of microorganism, including but not limited to, bacteria, viruses, archaea, fungi, protozoans, mycoplasma, prions, and parasitic organisms. The term microorganism encompasses both those organisms that are in and of themselves pathogenic to another organism (e.g., animals, including humans, and plants) and those organisms that produce agents that are pathogenic to another organism, while the organism itself is not directly pathogenic or infective to the other organism.

As used herein the term "pathogen," and grammatical equivalents, refers to an organism (e.g., biological agent), including microorganisms, that causes a disease state (e.g., infection, pathologic condition, disease, etc.) in another organism (e.g., animals and plants) by directly infecting the other organism, or by producing agents that causes disease in another organism (e.g., bacteria that produce pathogenic toxins and the like). "Pathogens" include, but are not limited to, viruses, bacteria, archaea, fungi, protozoans, mycoplasma, prions, and parasitic organisms.

The terms "bacteria" and "bacterium" refer to all prokaryotic organisms, including those within all of the phyla in the Kingdom Procaryotae. It is intended that the term encompass all microorganisms considered to be bacteria including *Mycoplasma*, *Chlamydia*, *Actinomyces*, *Streptomyces*, and *Rickettsia*. All forms of bacteria are included within this definition including cocci, bacilli, spirochetes, spheroplasts, protoplasts, etc.

As used herein, the term "fungi" is used in reference to eukaryotic organisms such as molds and yeasts, including dimorphic fungi.

As used herein the terms "disease" and "pathologic condition" are used interchangeably, unless indicated otherwise herein, to describe a deviation from the condition regarded as normal or average for members of a species or group (e.g., humans),

and which is detrimental to an affected individual under conditions that are not inimical to the majority of individuals of that species or group. Such a deviation can manifest as a state, signs, and/or symptoms (e.g., diarrhea, nausea, fever, pain, blisters, boils, rash, immune suppression, inflammation, etc.) that are associated with any impairment of the normal state of a subject or of any of its organs or tissues that interrupts or modifies the performance of normal functions. A disease or pathological condition may be caused by or result from contact with a microorganism (e.g., a pathogen or other infective agent (e.g., a virus or bacteria)), may be responsive to environmental factors (e.g., malnutrition, industrial hazards, and/or climate), may be responsive to an inherent defect of the organism (e.g., genetic anomalies) or to combinations of these and other factors.

The terms "host" or "subject," as used herein, refer to an individual to be treated by (e.g., administered) the compositions and methods of the present invention. Subjects include, but are not limited to, mammals (e.g., murines, simians, equines, bovines, porcines, canines, felines, and the like), and most preferably includes humans. In the context of the invention, the term "subject" generally refers to an individual who will be administered or who has been administered one or more compositions of the present invention (e.g., a composition for inducing an immune response).

As used herein, the terms "inactivating," "inactivation" and grammatical equivalents, when used in reference to a microorganism (e.g., a pathogen (e.g., a bacterium or a virus)), refer to the killing, elimination, neutralization and/or reducing of the capacity of the microorganism (e.g., a pathogen (e.g., a bacterium or a virus)) to infect and/or cause a pathological response and/or disease in a host. For example, in some embodiments, the present invention provides a composition comprising nanoemulsion (NE)-inactivated vaccinia virus (VV). Accordingly, as referred to herein, compositions comprising "NE-inactivated VV," "NE-killed V," "NE-neutralized V" or grammatical equivalents refer to compositions that, when administered to a subject, are characterized by the absence of, or significantly reduced presence of, VV replication (e.g., over a period of time (e.g., over a period of days, weeks, months, or longer)) within the host.

As used herein, the term "fusigenic" is intended to refer to an emulsion that is capable of fusing with the membrane of a microbial agent (e.g., a bacterium or bacterial spore). Specific examples of fusigenic emulsions are described herein.

As used herein, the term "lysogenic" refers to an emulsion (e.g., a nanoemulsion) that is capable of disrupting the membrane of a microbial agent (e.g., a virus (e.g., viral

envelope) or a bacterium or bacterial spore). In preferred embodiments of the present invention, the presence of a lysogenic and a fusigenic agent in the same composition produces an enhanced inactivating effect compared to either agent alone. Methods and compositions (e.g., for inducing an immune response (e.g., used as a vaccine) using this improved antimicrobial composition are described in detail herein.

The term "emulsion," as used herein, includes classic oil-in-water or water in oil dispersions or droplets, as well as other lipid structures that can form as a result of hydrophobic forces that drive apolar residues (e.g., long hydrocarbon chains) away from water and drive polar head groups toward water, when a water immiscible oily phase is mixed with an aqueous phase. These other lipid structures include, but are not limited to, unilamellar, paucilamellar, and multilamellar lipid vesicles, micelles, and lamellar phases. Similarly, the term "nanoemulsion," as used herein, refers to oil-in-water dispersions comprising small lipid structures. For example, in preferred embodiments, the nanoemulsions comprise an oil phase having droplets with a mean particle size of approximately 0.1 to 5 microns (e.g., 150 +/-25 nm in diameter), although smaller and larger particle sizes are contemplated. The terms "emulsion" and "nanoemulsion" are often used herein, interchangeably, to refer to the nanoemulsions of the present invention.

As used herein, the terms "contact," "contacted," "expose," and "exposed," when used in reference to a nanoemulsion and a live microorganism, refer to bringing one or more nanoemulsions into contact with a microorganism (e.g., a pathogen) such that the nanoemulsion inactivates the microorganism or pathogenic agent, if present. The present invention is not limited by the amount or type of nanoemulsion used for microorganism inactivation. A variety of nanoemulsion that find use in the present invention are described herein and elsewhere (e.g., nanoemulsions described in U.S. Pat. Apps. 20020045667 and 20040043041, and U.S. Pat. Nos. 6,015,832, 6,506,803, 6,635,676, and 6,559,189, each of which is incorporated herein by reference in its entirety for all purposes). Ratios and amounts of nanoemulsion (e.g., sufficient for inactivating the microorganism (e.g., virus inactivation)) and microorganisms (e.g., sufficient to provide an antigenic composition (e.g., a composition capable of inducing an immune response)) are contemplated in the present invention including, but not limited to, those described herein.

The term "surfactant" refers to any molecule having both a polar head group, which energetically prefers solvation by water, and a hydrophobic tail that is not well solvated by water. The term "cationic surfactant" refers to a surfactant with a cationic head group. The

term "anionic surfactant" refers to a surfactant with an anionic head group.

The terms "Hydrophile-Lipophile Balance Index Number" and "HLB Index Number" refer to an index for correlating the chemical structure of surfactant molecules with their surface activity. The HLB Index Number may be calculated by a variety of empirical formulas as described, for example, by Meyers, (See, e.g., Meyers, *Surfactant Science and Technology*, VCH Publishers Inc., New York, pp. 231-245 (1992)), incorporated herein by reference. As used herein where appropriate, the HLB Index Number of a surfactant is the HLB Index Number assigned to that surfactant in McCutcheon's Volume 1: Emulsifiers and Detergents North American Edition, 1996 (incorporated herein by reference). The HLB Index Number ranges from 0 to about 70 or more for commercial surfactants. Hydrophilic surfactants with high solubility in water and solubilizing properties are at the high end of the scale, while surfactants with low solubility in water that are good solubilizers of water in oils are at the low end of the scale.

As used herein the term "interaction enhancers" refers to compounds that act to enhance the interaction of an emulsion with a microorganism (e.g., with a cell wall of a bacteria (e.g., a Gram negative bacteria) or with a viral envelope (e.g., Vaccinia virus envelope)). Contemplated interaction enhancers include, but are not limited to, chelating agents (e.g., ethylenediaminetetraacetic acid (EDTA), ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), and the like) and certain biological agents (e.g., bovine serum albumin (BSA) and the like).

The terms "buffer" or "buffering agents" refer to materials, that when added to a solution, cause the solution to resist changes in pH.

The terms "reducing agent" and "electron donor" refer to a material that donates electrons to a second material to reduce the oxidation state of one or more of the second material's atoms.

The term "monovalent salt" refers to any salt in which the metal (e.g., Na, K, or Li) has a net 1+ charge in solution (i.e., one more proton than electron).

The term "divalent salt" refers to any salt in which a metal (e.g., Mg, Ca, or Sr) has a net 2+ charge in solution.

The terms "chelator" or "chelating agent" refer to any materials having more than one atom with a lone pair of electrons that are available to bond to a metal ion.

The term "solution" refers to an aqueous or non-aqueous mixture.

As used herein, the term "a composition for inducing an immune response" refers to

a composition that, once administered to a subject (e.g., once, twice, three times or more (e.g., separated by weeks, months or years)), stimulates, generates and/or elicits an immune response in the subject (e.g., resulting in total or partial immunity to a microorganism (e.g., pathogen) capable of causing disease). In preferred embodiments of the invention, the composition comprises a nanoemulsion and an immunogen. In further preferred embodiments, the composition comprising a nanoemulsion and an immunogen comprises one or more other compounds or agents including, but not limited to, therapeutic agents, physiologically tolerable liquids, gels, carriers, diluents, adjuvants, excipients, salicylates, steroids, immunosuppressants, immunostimulants, antibodies, cytokines, antibiotics, binders, fillers, preservatives, stabilizing agents, emulsifiers, and/or buffers. An immune response may be an innate (e.g., a non-specific) immune response or a learned (e.g., acquired) immune response (e.g. that decreases the infectivity, morbidity, or onset of mortality in a subject (e.g., caused by exposure to a pathogenic microorganism) or that prevents infectivity, morbidity, or onset of mortality in a subject (e.g., caused by exposure to a pathogenic microorganism)). Thus, in some preferred embodiments, a composition comprising a nanoemulsion and an immunogen is administered to a subject as a vaccine (e.g., to prevent or attenuate a disease (e.g., by providing to the subject total or partial immunity against the disease or the total or partial attenuation (e.g., suppression) of a sign, symptom or condition of the disease).

As used herein, the term "adjuvant" refers to any substance that can stimulate an immune response (e.g., a mucosal immune response). Some adjuvants can cause activation of a cell of the immune system (e.g., an adjuvant can cause an immune cell to produce and secrete a cytokine). Examples of adjuvants that can cause activation of a cell of the immune system include, but are not limited to, saponins purified from the bark of the Q. saponaria tree, such as QS21 (a glycolipid that elutes in the 21st peak with HPLC fractionation; Aquila Biopharmaceuticals, Inc., Worcester, Mass.); poly(di(carboxylatophenoxy)phosphazene (PCPP polymer; Virus Research Institute, USA); derivatives of lipopolysaccharides such as monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Inc., Hamilton, Mont.), muramyl dipeptide (MDP; Ribi) and threonyl-muramyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland); and Leishmania elongation factor (a purified Leishmania protein; Corixa Corporation, Seattle, Wash.). Traditional adjuvants are well known in the art and include, for example, aluminum phosphate or hydroxide salts

("alum"). In some embodiments, compositions of the present invention (e.g., comprising HIV or an immunogenic epitope thereof (e.g., gp120)) are administered with one or more adjuvants (e.g., to skew the immune response towards a Th1 or Th2 type response).

As used herein, the term "an amount effective to induce an immune response" (e.g.,
 5 of a composition for inducing an immune response), refers to the dosage level required (e.g., when administered to a subject) to stimulate, generate and/or elicit an immune response in the subject. An effective amount can be administered in one or more administrations (e.g., via the same or different route), applications or dosages and is not intended to be limited to a particular formulation or administration route.

) As used herein, the term "under conditions such that said subject generates an immune response" refers to any qualitative or quantitative induction, generation, and/or stimulation of an immune response (e.g., innate or acquired).

A used herein, the term "immune response" refers to a response by the immune system of a subject. For example, immune responses include, but are not limited to, a
 5 detectable alteration (e.g., increase) in Toll receptor activation, lymphokine (e.g., cytokine (e.g., Th1 or Th2 type cytokines) or chemokine) expression and/or secretion, macrophage activation, dendritic cell activation, T cell activation (e.g., CD4+ or CD8+ T cells), NK cell activation, and/or B cell activation (e.g., antibody generation and/or secretion). Additional examples of immune responses include binding of an immunogen (e.g., antigen (e.g.,
) immunogenic polypeptide)) to an MHC molecule and inducing a cytotoxic T lymphocyte ("CTL") response, inducing a B cell response (e.g., antibody production), and/or T-helper lymphocyte response, and/or a delayed type hypersensitivity (DTH) response against the antigen from which the immunogenic polypeptide is derived, expansion (e.g., growth of a population of cells) of cells of the immune system (e.g., T cells, B cells (e.g., of any stage of
 5 development (e.g., plasma cells), and increased processing and presentation of antigen by antigen presenting cells. An immune response may be to immunogens that the subject's immune system recognizes as foreign (e.g., non-self antigens from microorganisms (e.g., pathogens), or self-antigens recognized as foreign). Thus, it is to be understood that, as used herein, "immune response" refers to any type of immune response, including, but not limited
) to, innate immune responses (e.g., activation of Toll receptor signaling cascade) cell-mediated immune responses (e.g., responses mediated by T cells (e.g., antigen-specific T cells) and non-specific cells of the immune system) and humoral immune responses (e.g., responses mediated by B cells (e.g., via generation and secretion of antibodies into the

plasma, lymph, and/or tissue fluids). The term "immune response" is meant to encompass all aspects of the capability of a subject's immune system to respond to antigens and/or immunogens (e.g., both the initial response to an immunogen (e.g., a pathogen) as well as acquired (e.g., memory) responses that are a result of an adaptive immune response).

As used herein, the term "immunity" refers to protection from disease (e.g., preventing or attenuating (e.g., suppression) of a sign, symptom or condition of the disease) upon exposure to a microorganism (e.g., pathogen) capable of causing the disease. Immunity can be innate (e.g., non-adaptive (e.g., non-acquired) immune responses that exist in the absence of a previous exposure to an antigen) and/or acquired (e.g., immune responses that are mediated by B and T cells following a previous exposure to antigen (e.g., that exhibit increased specificity and reactivity to the antigen)).

As used herein, the term "immunogen" refers to an agent (e.g., a microorganism (e.g., bacterium, virus or fungus) or portion thereof (e.g., a protein antigen (e.g., gp120 or rPA))) that is capable of eliciting an immune response in a subject. In preferred embodiments, immunogens elicit immunity against the immunogen (e.g., microorganism (e.g., pathogen or a pathogen product)) when administered in combination with a nanoemulsion of the present invention.

As used herein, the term "pathogen product" refers to any component or product derived from a pathogen including, but not limited to, polypeptides, peptides, proteins, nucleic acids, membrane fractions, and polysaccharides.

As used herein, the term "enhanced immunity" refers to an increase in the level of adaptive and/or acquired immunity in a subject to a given immunogen (e.g., microorganism (e.g., pathogen)) following administration of a composition (e.g., composition for inducing an immune response of the present invention) relative to the level of adaptive and/or acquired immunity in a subject that has not been administered the composition (e.g., composition for inducing an immune response of the present invention).

As used herein, the terms "purified" or "to purify" refer to the removal of contaminants or undesired compounds from a sample or composition. As used herein, the term "substantially purified" refers to the removal of from about 70 to 90 %, up to 100%, of the contaminants or undesired compounds from a sample or composition.

As used herein, the terms "administration" and "administering" refer to the act of giving a composition of the present invention (e.g., a composition for inducing an immune response (e.g., a composition comprising a nanoemulsion and an immunogen)) to a subject.

Exemplary routes of administration to the human body include, but are not limited to, through the eyes (ophthalmic), mouth (oral), skin (transdermal), nose (nasal), lungs (inhalant), oral mucosa (buccal), ear, rectal, by injection (e.g., intravenously, subcutaneously, intraperitoneally, etc.), topically, and the like.

As used herein, the terms "co-administration" and "co-administering" refer to the administration of at least two agent(s) (e.g., a composition comprising a nanoemulsion and an immunogen and one or more other agents - e.g., an adjuvant) or therapies to a subject. In some embodiments, the co-administration of two or more agents or therapies is concurrent. In other embodiments, a first agent/therapy is administered prior to a second agent/therapy. In some embodiments, co-administration can be via the same or different route of administration. Those of skill in the art understand that the formulations and/or routes of administration of the various agents or therapies used may vary. The appropriate dosage for co-administration can be readily determined by one skilled in the art. In some embodiments, when agents or therapies are co-administered, the respective agents or therapies are administered at lower dosages than appropriate for their administration alone. Thus, co-administration is especially desirable in embodiments where the co-administration of the agents or therapies lowers the requisite dosage of a potentially harmful (e.g., toxic) agent(s), and/or when co-administration of two or more agents results in sensitization of a subject to beneficial effects of one of the agents via co-administration of the other agent. In other embodiments, co-administration is preferable to elicit an immune response in a subject to two or more different immunogens (e.g., microorganisms (e.g., pathogens)) at or near the same time (e.g., when a subject is unlikely to be available for subsequent administration of a second, third, or more composition for inducing an immune response).

As used herein, the term "topically" refers to application of a compositions of the present invention (e.g., a composition comprising a nanoemulsion and an immunogen) to the surface of the skin and/or mucosal cells and tissues (e.g., alveolar, buccal, lingual, masticatory, vaginal or nasal mucosa, and other tissues and cells which line hollow organs or body cavities).

In some embodiments, the compositions of the present invention are administered in the form of topical emulsions, injectable compositions, ingestible solutions, and the like. When the route is topical, the form may be, for example, a spray (e.g., a nasal spray), a cream, or other viscous solution (e.g., a composition comprising a nanoemulsion and an immunogen in polyethylene glycol).

The terms "pharmaceutically acceptable" or "pharmacologically acceptable," as used herein, refer to compositions that do not substantially produce adverse reactions (e.g., toxic, allergic or immunological reactions) when administered to a subject.

As used herein, the term "pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers including, but not limited to, phosphate buffered saline solution, water, and various types of wetting agents (e.g., sodium lauryl sulfate), any and all solvents, dispersion media, coatings, sodium lauryl sulfate, isotonic and absorption delaying agents, disintegrants (e.g., potato starch or sodium starch glycolate), polyethylene glycol, and the like.. The compositions also can include stabilizers and preservatives. Examples of carriers, stabilizers and adjuvants have been described and are known in the art (See e.g., Martin, Remington's Pharmaceutical Sciences, 15th Ed., Mack Publ. Co., Easton, Pa. (1975), incorporated herein by reference).

As used herein, the term "pharmaceutically acceptable salt" refers to any salt (e.g., obtained by reaction with an acid or a base) of a composition of the present invention that is physiologically tolerated in the target subject. "Salts" of the compositions of the present invention may be derived from inorganic or organic acids and bases. Examples of acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, sulfonic, naphthalene-2-sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compositions of the invention and their pharmaceutically acceptable acid addition salts.

Examples of bases include, but are not limited to, alkali metal (e.g., sodium) hydroxides, alkaline earth metal (e.g., magnesium) hydroxides, ammonia, and compounds of formula NW_4^+ , wherein W is C_{1-4} alkyl, and the like.

Examples of salts include, but are not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, chloride, bromide, iodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, undecanoate, and the

like. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na^+ , NH_4^+ , and NW_4^+ (wherein W is a C_{1-4} alkyl group), and the like. For therapeutic use, salts of the compounds of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases that are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

For therapeutic use, salts of the compositions of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases that are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable composition.

As used herein, the term "at risk for disease" refers to a subject that is predisposed to experiencing a particular disease. This predisposition may be genetic (e.g., a particular genetic tendency to experience the disease, such as heritable disorders), or due to other factors (e.g., environmental conditions, exposures to detrimental compounds present in the environment, etc.). Thus, it is not intended that the present invention be limited to any particular risk (e.g., a subject may be "at risk for disease" simply by being exposed to and interacting with other people), nor is it intended that the present invention be limited to any particular disease.

"Nasal application", as used herein, means applied through the nose into the nasal or sinus passages or both. The application may, for example, be done by drops, sprays, mists, coatings or mixtures thereof applied to the nasal and sinus passages.

"Vaginal application", as used herein, means applied into or through the vagina so as to contact vaginal mucosa. The application may contact the urethra, cervix, fornix, uterus or other area surrounding the vagina. The application may, for example, be done by drops, sprays, mists, coatings, lubricants or mixtures thereof applied to the vagina or surrounding tissue.

As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of immunogenic agents (e.g., compositions comprising a nanoemulsion and an immunogen), such delivery systems include systems that allow for the storage, transport, or delivery of immunogenic agents and/or supporting materials (e.g., written instructions for using the materials, etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant immunogenic agents (e.g., nanoemulsions) and/or supporting materials. As used herein, the term "fragmented kit"

refers to delivery systems comprising two or more separate containers that each contain a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain a composition comprising a nanoemulsion and an immunogen for a particular use, while a second container contains a second agent (e.g., an antibiotic or spray applicator). Indeed, any delivery system comprising two or more separate containers that each contains a subportion of the total kit components are included in the term "fragmented kit." In contrast, a "combined kit" refers to a delivery system containing all of the components of an immunogenic agent needed for a particular use in a single container (e.g., in a single box housing each of the desired components). The term "kit" includes both fragmented and combined kits.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for the stimulation of immune responses. Specifically, the present invention provides methods of inducing an immune response to an agent (e.g., a bacteria or virus) in a subject (e.g., a human subject) and compositions useful in such methods (e.g., a nanoemulsion comprising a bacteria or virus or a component thereof). Compositions and methods of the present invention find use in, among other things, clinical (e.g. therapeutic and preventative medicine (e.g., vaccination)) and research applications.

Several pathogenic microorganisms initiate infection by attaching to mucosal epithelial cells lining the gastro-intestinal, oropharyngeal, respiratory or genito-urinary tracts. Some pathogens, such as influenza virus, Bordetella pertussis, or Vibrio cholerae, remain at or within the mucosal tissue, while others, such as Salmonella typhi or hepatitis A virus, possess mechanisms permitting penetration into deeper tissues and spread systemically. Specific and non-specific defense mechanisms of the mucous membranes provide first line protection against both types of pathogen. Non-specific effectors include, for example, resident macrophages, antimicrobial peptides, lactoferrin and lysozyme, extremes of pH, bile acids, digestive enzymes, mucus, shedding of epithelial cells, flushing mechanisms (peristalsis, ciliary beating, micturation, etc) and competition from local flora. However, successful pathogens have generally evolved means to survive the non-specific defenses present at the site they infect and it is the secretory immune system that plays a major role in protecting against diseases caused by a number of bacterial and viral

pathogens, and is a major effector against pathogens that are restricted to mucosal surfaces. For organisms that spread systemically, both local and systemic immune responses are likely needed for optimum immunity.

Existing forms of orthopox (e.g., smallpox, cowpox, monkeypox, gerbilpox, camelpox, and others) vaccines are obsolete. For example, all current, licensed smallpox vaccines that were used to achieve smallpox eradication are based on live Vaccinia virus (VV) obtained from infected calf's skin and lymph nodes. Although these vaccines confer long-lasting immunity against several different orthopox viruses, the materials they are made from do not meet current standards for human vaccines. Furthermore, these vaccines also produce infectious skin pustules (pox) and infrequent but severe side reactions limiting their use in individuals (and their close contacts) with immunodeficiency, eczema, atopic dermatitis, or heart disease (See, e.g., Eichner and Schwehm, *Epidemiology*. 2004, 15(3):258-60).

It is anticipated that future applications for orthopox vaccines (e.g., smallpox, cowpox, monkeypox, gerbilpox, camelpox, and others) would be a response to either a bioterrorist attack or outbreaks of other orthopox infections, such as monkeypox or cowpox (See, e.g., Edghill-Smith et al., *Nature Med.* 2005, 11; 740-747). Because of this, the risk/benefit ratio of vaccination will require that new smallpox vaccines place a high priority on safety. Additionally, for use in emergent public health situations, it will be imperative to replace vaccines administered by scarification with a rapidly administered vaccine (e.g., a mucosal vaccine). Such administration may, in addition to providing a vaccination and long-term immune protection, may provide a quick, on-site generator of immune responses (e.g., that reduce infection, symptoms and/or time course of disease).

Accordingly, the present invention provides methods of inducing an immune response to orthopox viruses (e.g., vaccinia virus) in a subject (e.g., a human subject) and compositions useful in such methods (e.g., a nanoemulsion comprising an orthopox virus (e.g., vaccinia virus (VV))). In preferred embodiments, methods of inducing an immune response provided by the present invention are used for vaccination. Due to the rate of adverse events with existing orthopox (e.g., smallpox) vaccines, the present invention provides a significant improvement in orthopox (e.g., small pox) vaccination safety without compromising vaccine efficiency.

For example, the present invention describes the development of immunity (e.g., VV immunity) in a subject after mucosal administration (e.g., mucosal vaccination) of a unique

type of inactivated orthopox virus (e.g., VV) preparation identified and characterized during development of the present invention. Nanoemulsion (NE), a surface-active antimicrobial material, was mixed with highly purified, cell culture-derived VV, resulting in a formulation (e.g., NE-killed VV composition) that is stable at room temperature (e.g., in some embodiments, for more than 2 weeks, more preferably more than 3 weeks, even more preferably more than 4 weeks, and most preferably for more than 5 weeks) and that can be used to induce an immune response against orthopox viruses (e.g., VV) in a subject (e.g., that can be used either alone or as an adjuvant for inducing an anti-VV immune response).

Mucosal administration of a composition comprising NE and VV (e.g., NE-killed VV) to a subject resulted in high-titer mucosal and systemic antibody responses and specific Th1 cellular immunity (See, e.g., Examples 2-4, and 6). Further, all animals were fully protected against an nasal instillation challenge with 10x LD₅₀ VV (See, e.g., Example 7, Figure 5). In the vaccinated animals, infection was completely prevented or was of a low level and self-limiting and infection resolved in four to five days. In contrast, all naive animals died within this time period. Subsequent re-challenge of immunized mice with a 100x LD of VV-WR validated protective immunity. Mice administered even a single dose of a composition comprising NE-killed VV developed significant serum concentrations of anti-VV IgG 10 to 12 weeks after administration (See, e.g., Example 2). This level of response is comparable to the results obtained in Balb/c mice immunized by intramuscular injection with live VV Wyeth at similar time point (See, e.g., Coulibaly et al., Virology, 2005; 341; 91-101). Thus, in some embodiments, the present invention provides that a single administration (e.g., mucosal administration) of a composition comprising NE-killed VV is sufficient to induce a protective immune response in a subject (e.g., protective immunity (e.g., mucosal and systemic immunity)). In some embodiments, a subsequent administration (e.g., one or more boost administrations subsequent to a primary administration) to a subject provides the induction of an enhanced immune response to VV in the subject. Thus, the present invention demonstrates that administration of a composition comprising NE-killed VV to a subject provides protective immunity against smallpox.

In contrast, intranasal instillations of formalin-killed VV with or without nanoemulsion produced inconsistent and low antibody responses, which did not augment even after a third immunization (See, e.g., Example 2). A similar pattern of neutralizing activity was also detected in serum and bronchoalveolar lavage (BAL), with neutralizing activity being absent in mice mucosally vaccinated with formalin-killed virus. Neutralizing

activity was not detected in BAL of animals vaccinated with either IP or SQ injections of a live virus. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, NE treatment (e.g., neutralization of an orthopox virus (e.g., VV) with a NE of the present invention) preserves important viral neutralizing epitopes (e.g., recognizable by a subject's immune system), stabilizing their hydrophobic and hydrophilic components in the oil and water interface of the emulsion (e.g., thereby providing one or more immunogens (e.g., stabilized antigens) against which a subject can mount an immune response). In other embodiments, because NE formulations are known to penetrate the mucosa through pores, they may carry viral proteins to the submucosal location of dendritic cells (e.g., thereby initiating and/or stimulating an immune response).

Dendritic cells avidly phagocytose NE oil droplets and this could provide a means to internalize antigenic proteins for antigen presentation. While other vaccines rely on inflammatory toxins or other immune stimuli for adjuvant activity (See, e.g., Holmgren and Czerkinsky, *Nature Med.* 2005, 11; 45-53), NEs have not been shown to be inflammatory when placed on the skin or mucous membranes in studies on animals and in humans. Thus, although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, a composition comprising a NE of the present invention (e.g., a composition comprising NE-inactivated orthopox virus (e.g., VV)) may act as a "physical" adjuvant (e.g., that transports and/or presents orthopox antigens (e.g., Vaccinia proteins) to the immune system. In some preferred embodiments, mucosal administration of a composition of the present invention generates mucosal as well as systemic immunity (e.g., signs of mucosal immunity (e.g., generation of IgA antibody titers).

Both cellular and humoral immunity play a role in protection against orthopoxviruses, and both were induced with the NE formulations (See, e.g., Examples 2-4, and 6). Vaccinia-specific antibody titers are considered important for the estimate of protective immunity in human subjects and in animal models of vaccination (See, e.g., Hammarlund et al, *Nat. Med.* 2003, 9; 1131-1137). Several studies have identified proteins important for the elicitation of neutralizing antibodies (See, e.g., Galmiche et al, *Virology*, 1999, 254; 71-80; Hooper et al, *Virology*, 2003, 306; 181-195). A recent trial of dilutions of the licensed smallpox vaccine (Dryvax) in human volunteers, confirmed that pustule formation strongly correlated with development of both specific antibodies and induction of

cytotoxic T lymphocytes (CTL) and elevated $\text{INF-}\gamma$ T cell responses (See, e.g., Greenberg et al, 2005, 365; 398-409). Induction of $\text{INF-}\gamma$ is suggestive of activation of specific MHC class I-restricted CD8^+ T cells. These types of cells have been implicated in the recognition and clearance of Vaccinia infected cells, and for maintenance of immunity after vaccination (See, e.g., Earl et al, Nature, 2004; 482; 182-185; Hammarlund et al, Nat. Med. 2003, 9; 1131-1137; Edghill-Smith et al, Nature Med. 2005, 11; 740-747).

Thus, in some embodiments, administration (e.g., mucosal administration) of a composition of the present invention (e.g., NE-killed orthopox virus (e.g., VV) to a subject results in the induction of both humoral (e.g., development of specific antibodies) and cellular (e.g., cytotoxic T lymphocyte) immune responses (e.g., against the orthopox virus). In some preferred embodiments, a composition of the present invention (e.g., NE-killed orthopox virus (e.g., VV) is used as a smallpox vaccine.

Furthermore, in preferred embodiments, a composition of the present invention (e.g., NE-killed orthopox virus (e.g., VV) induces (e.g., when administered to a subject) both systemic and mucosal immunity. Thus, in some preferred embodiments, administration of a composition of the present invention to a subject results in protection against an exposure (e.g., a lethal mucosal exposure) to an orthopox virus (e.g., VV). Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, mucosal administration (e.g., vaccination) provides protection against orthopox virus (e.g., VV) infection (e.g., that initiates at a mucosal surface). Although it has heretofore proven difficult to stimulate secretory IgA responses and protection against pathogens that invade at mucosal surfaces (See, e.g., Mestecky et al, Mucosal Immunology. 3ed edn. (Academic Press, San Diego, 2005)), the present invention provides compositions and methods for stimulating mucosal immunity (e.g., a protective IgA response) from a pathogen in a subject.

In some embodiments, the present invention provides a composition (e.g., a NE-inactivated orthopox virus (e.g., VV) formulation) to serve as a mucosal vaccine. This material can easily be produced from purified virus (See, e.g., Example 1), and induces both mucosal and systemic immunity (See, e.g., Examples 2-7). The ability to produce this formulation rapidly and administer it via nasal instillation provides a vaccine that can be used in large-scale outbreaks or emergent situations.

In some embodiments, the present invention provides compositions for generating an

immune response and methods of using the same (e.g., for use as a vaccine). In some preferred embodiments, a composition for generating an immune response comprises a NE and an immunogen (e.g., an orthopox virus (e.g., VV) inactivated by the nanoemulsion). When administered to a subject, a composition of the present invention stimulates an immune response against the immunogen (e.g., orthopox virus (e.g., VV)) within the subject. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, generation of an immune response (e.g., resulting from administration of a composition comprising a nanoemulsion and an orthopox virus (e.g., VV)) provides total or partial immunity to the subject (e.g., from signs, symptoms or conditions of a disease (e.g., smallpox)). Without being bound to any specific theory, protection and/or immunity from disease (e.g., the ability of a subject's immune system to prevent or attenuate (e.g., suppress) a sign, symptom or condition of disease) upon exposure to a nanoemulsion comprising an orthopox virus (e.g., VV) is due to adaptive (e.g., acquired) immune responses (e.g., immune responses mediated by B and T cells following exposure to a NE comprising an orthopox virus (e.g., VV) of the present invention (e.g., immune responses that exhibit increased specificity and reactivity to an orthopox virus (e.g., VV)).

In some embodiments, a NE comprising an immunogen (e.g., an orthopox virus (e.g., VV) inactivated by the NE) is administered alone. In some embodiments, a composition comprising a NE and an immunogen (e.g., an orthopox virus (e.g., VV) inactivated by the NE) comprises one or more other agents (e.g., a pharmaceutically acceptable carrier, adjuvant, excipient, and the like). In some embodiments, a composition for stimulating an immune response of the present invention is administered in a manner to induce a humoral immune response. In some embodiments, a composition for stimulating an immune response of the present invention is administered in a manner to induce a cellular (e.g., cytotoxic T lymphocyte) immune response, rather than a humoral response. In some embodiments, a composition comprising a NE and an immunogen of the present invention induces both a cellular and humoral immune response.

The present invention is not limited by the type of NE utilized (e.g., in an immunogenic composition comprising an immunogen. Indeed, a variety of NE compositions are contemplated to be useful in the present invention.

For example, in some embodiments, a nanoemulsion (e.g., for inactivation of an

orthopox virus (e.g., VV)) comprises (i) an aqueous phase; (ii) an oil phase; and at least one additional compound. In some embodiments of the present invention, these additional compounds are admixed into either the aqueous or oil phases of the composition. In other embodiments, these additional compounds are admixed into a composition of previously emulsified oil and aqueous phases. In certain of these embodiments, one or more additional compounds are admixed into an existing emulsion composition immediately prior to its use. In other embodiments, one or more additional compounds are admixed into an existing emulsion composition prior to the compositions immediate use.

Additional compounds suitable for use in a nanoemulsion of the present invention include, but are not limited to, one or more organic, and more particularly, organic phosphate based solvents, surfactants and detergents, cationic halogen containing compounds, germination enhancers, interaction enhancers, food additives (e.g., flavorings, sweeteners, bulking agents, and the like) and pharmaceutically acceptable compounds. Certain exemplary embodiments of the various compounds contemplated for use in the compositions of the present invention are presented below.

Several pathogenic microorganisms initiate infection by attaching to mucosal epithelial cells lining the gastro-intestinal, oropharyngeal, respiratory or genito-urinary tracts. Some pathogens, such as influenza virus, *Bordetella pertussis*, or *Vibrio cholerae*, remain at or within the mucosal tissue, while others, such as *Salmonella typhi* or hepatitis A virus, possess mechanisms permitting penetration into deeper tissues and spread systemically. Specific and non-specific defense mechanisms of the mucous membranes provide first line protection against both types of pathogen. Non-specific effectors include resident macrophages, antimicrobial peptides, lactoferrin and lysozyme, extremes of pH, bile acids, digestive enzymes, mucus, shedding of epithelial cells, flushing mechanisms (peristalsis, ciliary beating, micturation, etc) and competition from local flora. However, successful pathogens have generally evolved means to survive the non-specific defenses present at the site they infect and it is the secretory immune system which plays a major role in protecting against diseases caused by a number of bacterial and viral pathogens, and is probably a major effector against pathogens that are restricted to mucosal surfaces. For organisms that spread systemically, both local and systemic immune responses are desirable for optimum immunity.

Anthrax is an infectious bacterial disease caused by *Bacillus anthracis*. It occurs most commonly in wild and domestic herbivores (sheep, goats, camels, antelope, cattle, etc.)

but may also occur in humans. Infection can occur by cutaneous exposure, by ingestion (gastrointestinal anthrax), or by inhalation (pulmonary anthrax). 95% of anthrax infections in humans occur by cutaneous infection, either from contact with unvaccinated, infected animals in an agricultural setting, or by handling contaminated animal products (meat, leather, hides, hair, wool, etc.) in an industrial setting.

Cutaneous anthrax is fatal in about 20% of cases if untreated, but it can usually be overcome with appropriate antimicrobial therapy. Inhalation or gastrointestinal anthrax infection is much more serious and much more difficult to treat. Inhalation anthrax results in respiratory shock and is fatal in 90%-100% of cases; gastrointestinal anthrax results in severe fever, nausea and vomiting, resulting in death in 25%-75% of cases.

An effective vaccine against anthrax was developed in the United States in the 1950s and 1960s, and a vaccine was approved by the FDA in 1970.

The threat of airborne transmission of anthrax remains at historical highs as *B. anthracis* has been identified as a possible agent for biological warfare. Whereas historically only individuals at high risk, such as veterinarians, livestock handlers, wool shearers, abbatoire workers, etc., needed to consider being vaccinated, the threat to military personnel of the possibility of biological weapons deployment caused the United States military to adopt a sweeping anthrax vaccination program in 1997, under which it was intended to administer the anthrax vaccine to 2.4 million military personnel in all branches of service. (See, e.g., Secretary of Defense, Memorandum for Secretaries of the Military Departments et al., May 18, 1998, Implementation of the Anthrax Vaccination Program for the Total Force).

The only mass produced anthrax vaccine, Anthrax Vaccine Adsorbed (or AVA, commercial name BIOTHRAX), is a noninfectious sterile filtrate of an attenuated strain of *B. anthracis*, adsorbed to aluminum hydroxide (alum) adjuvant, with $\leq 0.02\%$ formaldehyde and 0.0025% benzethonium chloride added. (See, e.g., Friedlander et al., JAMA, 282(22):2104-2106 (1999)). The course of vaccination consists of six subcutaneous injections of 0.5 mL doses of vaccine over eighteen months, with annual boosters to maintain immunity. This vaccination is believed to provide immunity that is 90%-100% effective against aerosol anthrax challenge, based on animal studies and incidental human data. (See, e.g., Friedlander et al, supra).

While the AVA is moderately effective, the vaccine strain employed, a non-proteolytic, non-capsulated mutant strain of *B. anthracis*, V770-NP1-R, has several

disadvantageous characteristics: Despite its mutations, the strain retains a sporogenic and fully toxogenic phenotype, and use of the whole strain in vaccine production results in lot-to-lot variability in levels of protective antigen (PA), as well as inclusion of PA degradation products and other bacterial products (See, e.g., Farchaus, J., et al., *Applied & Environmental Microbiol.*, 64(3):982-991 (1998)). In addition, side effects reported from administration range from the common injection site swelling and tenderness, to systemic reactions (malaise, lassitude, fever, chills) as well as hair loss, muscle aches, chronic fatigue, aching teeth and gums, thick saliva, burn-like skin reactions, rapid weight loss, blackouts, and at least one death. (See, e.g., *Chicago Tribune*, *Mysterious illnesses strike some gulf vets*, Mar. 26, 1992, p.2; *The Washington Post*, *The Nation in Brief*, Sep. 29, 2000, Section A, p. 34). There exist some who claim the anthrax vaccine is contaminated with squalene (See, e.g., Garret, L., *Big Battle Over Vaccine: Detractors Say Immunization for Anthrax Hazardous; Pentagon Says No*, *The Beacon Journal (Akron)*, Sunday Jul. 4, 1999, Section B, p. 1), and has resulted in hundreds of military personnel refusing to be vaccinated (See, e.g., Graham, B., *Some in Military Fear Anthrax Inoculation Side Effects*, *The Plain Dealer (Cleveland)*, Nov. 26, 1998, Section: National, p. 6E; *Air Force Reserve Pilots Quitting Due to Vaccine*, *The Plain Dealer (Cleveland)*, Feb. 27, 1999, Section: National, p. 6A). Military personnel ranked as high as major have accepted court-martial and dismissal from military service rather than accept the anthrax vaccine. (See, e.g., Eskenazi, M., *How Anthrax Causes Early Retirement*, *TIME.com*, Mar. 31, 2000.)

Thus, a great need exists for an improved composition for immunization against anthrax, and other diseases caused by bacteria of the genus *Bacillus*, that is effective to raise an immune response against *B. anthracis*. Such a composition would ideally be formulated without contaminants (e.g., capable of generating unwanted side effects) and would be effective without a need for a long course of vaccination.

Accordingly, the present invention provides methods of inducing an immune response to bacteria of the genus *Bacillus* (e.g., *B. anthracis*) in a subject (e.g., a human subject) and compositions useful in such methods (e.g., a nanoemulsion comprising bacteria or bacterial components (e.g., isolated or recombinant proteins) of the genus *Bacillus* (e.g., *B. anthracis*)). In preferred embodiments, methods of inducing an immune response provided by the present invention are used for vaccination. Due to the rate of adverse events with existing *Bacillus* (e.g., *B. anthracis*) vaccines, the present invention provides a significant improvement in *Bacillus* (e.g., *B. anthracis*) vaccination safety without

compromising vaccine efficacy.

For example, the present invention describes the development of immunity (e.g., *B. anthracis* immunity) in a subject after mucosal administration (e.g., mucosal vaccination) with a composition comprising a nanoemulsion and an immunogenic protein from *B. anthracis* (e.g., rPA) generated and characterized during development of the present invention (See Examples 8-16). Nanoemulsion (NE), a surface-active antimicrobial material, was mixed with recombinant protective antigen (rPA), resulting in an immunogenic composition comprising NE and rPA that is stable at room temperature (e.g., in some embodiments, for more than 2 weeks, more preferably more than 3 weeks, even more preferably more than 4 weeks, and most preferably for more than 5 weeks) and that can be used to induce an immune response against *B. anthracis* in a subject (e.g., that can be used either alone or as an adjuvant for inducing an anti- *B. anthracis* immune response).

Mucosal administration of a composition comprising NE and rPA to a subject resulted in high-titer mucosal and systemic antibody responses and specific Th1 cellular immunity (See, e.g., Examples 11-12, 14-16). Further, serum from mice immunized intranasally with a composition comprising NE and rPA was capable of neutralizing binding of PA to its receptor (ATR receptor) (See Example 13). Mice administered two doses and guinea pigs administered just a single dose of a composition comprising NE and rPA developed significant serum concentrations of anti-rPA IgG after administration (See, e.g., Example 11). Moreover, mice administered this composition generated mucosal immune responses (e.g., IgA antibodies toward rPA) (See Example 12).

Thus, in some embodiments, the present invention provides that administration (e.g., mucosal administration) of a composition comprising NE and a *B. anthracis* immunogen (e.g., rPA) is sufficient to induce a protective immune response against *B. anthracis* in a subject (e.g., protective immunity (e.g., mucosal and systemic immunity)). In some embodiments, a subsequent administration (e.g., one or more boost administrations subsequent to a primary administration) to a subject provides the induction of an enhanced immune response to *B. anthracis* in the subject. Thus, the present invention demonstrates that administration of a composition comprising NE and a *B. anthracis* immunogen (e.g., rPA) to a subject provides protective immunity against anthrax (e.g., via a durable anti-PA IgG response).

In contrast, intranasal instillations of NE alone or NE with CpG adjuvant was not able to induce an immune response against *B. anthracis* (See Examples 11-13).

Furthermore, administration of rPA alone (e.g., in saline) did not induce significant IgG or IgA antibody production in mice. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, combining a NE and an immunogenic protein, rPA, from *B. anthracis* stabilizes the rPA (See Example 9) and provides the proper environment for generation of an immune response. In other embodiments, because NE formulations are known to penetrate the mucosa through pores, they may carry immunogenic proteins to the submucosal location of dendritic cells (e.g., thereby initiating and/or stimulating an immune response). In some embodiments, when a NE is used to inactivate bacteria of the genus *Bacillus* (e.g., *B. anthracis*) combining the bacteria and the NE preserves important immunogenic epitopes (e.g., recognizable by a subject's immune system), stabilizing their hydrophobic and hydrophilic components in the oil and water interface of the emulsion (e.g., thereby providing one or more immunogens (e.g., stabilized antigens) against which a subject can mount an immune response).

Dendritic cells avidly phagocytose NE oil droplets and this could provide a means to internalize immunogenic proteins for antigen presentation. While other vaccines rely on inflammatory toxins or other immune stimuli for adjuvant activity (See, e.g., Holmgren and Czerkinsky, Nature Med. 2005, 11; 45-53), NEs have not been shown to be inflammatory when placed on the skin or mucous membranes in studies on animals and in humans. Thus, although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, a composition comprising a NE of the present invention (e.g., a composition comprising NE and one or more *Bacillus* proteins (e.g., rPA) may act as a "physical" adjuvant (e.g., that transports and/or presents *Bacillus* proteins to the immune system (e.g., See Example 10)). In some preferred embodiments, mucosal administration of a composition of the present invention generates mucosal as well as systemic immunity (e.g., signs of mucosal immunity (e.g., generation of IgA antibody titers).

Both cellular and humoral immunity play a role in protection against *Bacillus* (e.g., *B. anthracis*), and both were induced with the NE formulations (See, e.g., Examples 11-16).

Thus, in some embodiments, administration (e.g., mucosal administration) of a composition of the present invention to a subject results in the induction of both humoral (e.g., development of specific antibodies) and cellular (e.g., cytotoxic T lymphocyte) immune responses (e.g., against *Bacillus* proteins). In some preferred embodiments, a composition

of the present invention (e.g., a composition comprising a NE and *Bacillus* proteins (e.g., rPA)) is used as an anthrax vaccine.

Furthermore, in preferred embodiments, a composition of the present invention induces (e.g., when administered to a subject) both systemic and mucosal immunity. Thus, in some preferred embodiments, administration of a composition of the present invention to a subject results in protection against an exposure (e.g., a lethal mucosal exposure) to *B. anthracis* (See Examples 8 and 9). Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, mucosal administration (e.g., vaccination) provides protection against *Bacillus* infection (e.g., that initiates at a mucosal surface). Although it has heretofore proven difficult to stimulate secretory IgA responses and protection against pathogens that invade at mucosal surfaces (See, e.g., Mestecky et al, Mucosal Immunology. 3rd Edn. (Academic Press, San Diego, 2005)), the present invention provides compositions and methods for stimulating mucosal immunity (e.g., a protective IgA response) from a pathogen in a subject.

In some embodiments, the present invention provides a composition (e.g., a composition comprising a NE and a *B. anthracis* immunogen (e.g., rPA)) to serve as a mucosal vaccine. This material can easily be produced with NE and recombinant protein (See, e.g., Example 1), and induces both mucosal and systemic immunity (See, e.g., Examples 11-16). The ability to produce this formulation rapidly and administer it via nasal instillation provides a vaccine that can be used in large-scale outbreaks or emergent situations.

In some preferred embodiments, the present invention provides a composition for generating an immune response comprising a NE and an immunogen (e.g., a purified, isolated or synthetic *Bacillus* protein or derivative or analogue thereof; or, bacteria of the genus *Bacillus* inactivated by the nanoemulsion). When administered to a subject, a composition of the present invention stimulates an immune response against the immunogen within the subject. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, generation of an immune response (e.g., resulting from administration of a composition comprising a nanoemulsion and a recombinant *Bacillus* protein) provides total or partial immunity to the subject (e.g., from signs, symptoms or conditions of a disease (e.g., anthrax)). Without being bound to any

specific theory, protection and/or immunity from disease (e.g., the ability of a subject's immune system to prevent or attenuate (e.g., suppress) a sign, symptom or condition of disease) upon exposure to an immunogenic composition of the present invention is due to adaptive (e.g., acquired) immune responses (e.g., immune responses mediated by B and T cells following exposure to a NE comprising a recombinant *Bacillus* protein of the present invention (e.g., immune responses that exhibit increased specificity and reactivity to *Bacillus*).

In some embodiments, a NE comprising an immunogen (e.g., a recombinant *Bacillus* protein) is administered alone. In some embodiments, a composition comprising a NE and an immunogen (e.g., a recombinant *Bacillus* protein) comprises one or more other agents (e.g., a pharmaceutically acceptable carrier, adjuvant, excipient, and the like). In some embodiments, a composition for stimulating an immune response of the present invention is administered in a manner to induce a humoral immune response. In some embodiments, a composition for stimulating an immune response of the present invention is administered in a manner to induce a cellular (e.g., cytotoxic T lymphocyte) immune response, rather than a humoral response. In some embodiments, a composition comprising a NE and an immunogen of the present invention induces both a cellular and humoral immune response.

The present invention is not limited by the type of NE utilized (e.g., in an immunogenic composition comprising an immunogen). Indeed, a variety of NE compositions are contemplated to be useful in the present invention.

For example, in some embodiments, a nanoemulsion comprises (i) an aqueous phase; (ii) an oil phase; and at least one additional compound. In some embodiments of the present invention, these additional compounds are admixed into either the aqueous or oil phases of the composition. In other embodiments, these additional compounds are admixed into a composition of previously emulsified oil and aqueous phases. In certain of these embodiments, one or more additional compounds are admixed into an existing emulsion composition immediately prior to its use. In other embodiments, one or more additional compounds are admixed into an existing emulsion composition prior to the compositions immediate use.

Additional compounds suitable for use in a nanoemulsion of the present invention include, but are not limited to, one or more organic, and more particularly, organic phosphate based solvents, surfactants and detergents, cationic halogen containing compounds, germination enhancers, interaction enhancers, food additives (e.g., flavorings,

sweeteners, bulking agents, and the like) and pharmaceutically acceptable compounds. Certain exemplary embodiments of the various compounds contemplated for use in the compositions of the present invention are presented below.

The HIV envelope glycoprotein gp120 is the viral protein that is used for attachment to the host cell. This attachment is mediated by the binding to two surface molecules of helper T cells and macrophages, known as CD4 and one of the two chemokine receptors CCR-4 or CXCR-5. The gp120 protein is first expressed as a larger precursor molecule (gp 160), which is then cleaved post-translationally to yield gp120 and gp41. The gp120 protein is retained on the surface of the virion by linkage to the gp41 molecule, which is inserted into the viral membrane.

The gp120 protein is the principal target of neutralizing antibodies. The most immunogenic regions of the gp120 proteins (V3 loop) are also the most variable parts of the protein. The gp120 protein also contains epitopes that are recognized by cytotoxic T lymphocytes (CTL). These effector cells are able to eliminate virus-infected cells, and therefore constitute a second major antiviral immune mechanism. In contrast to the target regions of neutralizing antibodies some CTL epitopes appear to be relatively conserved among different HIV strains. For this reason gp120 and gp 160 are considered to be useful antigenic components in vaccines that aim at eliciting cell-mediated immune responses (particularly CTL).

Several types of gp120 immunogenic antigens have been developed: (1) purified gp120 derived from HIV-infected tissue culture cells (referred to herein as "viral-derived gp120"); (2) gp120 made in cells infected with recombinant viruses, such as vaccinia or baculovirus (referred to herein as "live-virus-vector-derived gp120 and gp160"); (3) recombinant gp120 made in mammalian cells (referred to herein as "recombinant mammalian gp120"); (4) recombinant denatured polypeptides that represent all or various portions of gp120 and gp41 (referred to herein as "recombinant denatured antigens"); and (5) peptides that represent small segments of gp120 and gp41 (referred to herein as "peptides").

In general, each of these immunogenic antigens are highly immunogenic as adjuvanted in a variety of species. They have generated antibodies capable of neutralizing the homologous isolate of HIV-1. Levels of neutralization have not (in general) reached the level of neutralizing titer found in infected humans and there has been much difficulty generating an immunogenic composition that generates immunity to more than one strain of

HIV (e.g., other than the strain from which the immunogenic antigen was derived).

Another factor that has been particularly difficult to overcome when preparing HIV-1 vaccines is sequence diversity. HIV-1 and HIV-2 are characterized by having a very high level of sequence diversity that is most pronounced in the gp120 portion of the envelope. This sequence diversity is clustered in regions known as hypervariable regions. Many have proposed using a vaccine cocktail, comprising antigenic substances derived from a variety of HIV isolates, to provide protection against a broad range infective sources. The present invention is well suited for delivery of a composition comprising a variety of HIV antigenic substances derived from a variety of HIV isolates.

Thus, there remains a need for immunogenic substances capable of inducing neutralizing antibodies against HIV, preferably using a single source material that induces neutralizing antibodies against a variety of field isolates of HIV. Furthermore, substances capable of inducing both systemic as well as mucosal immunity to HIV would be highly desirable, as one of the surfaces most commonly exposed to HIV in humans is vaginal mucosa.

Accordingly, the present invention provides methods of inducing an immune response to HIV in a subject (e.g., a human subject) and compositions useful in such methods (e.g., a nanoemulsion comprising HIV or HIV components (e.g., isolated or recombinant HIV proteins). In some embodiments, methods of inducing an immune response provided by the present invention are used for vaccination. Due to the rate of adverse events with existing HIV vaccines, the present invention provides a significant improvement in HIV vaccination safety without compromising vaccine efficacy.

For example, the present invention describes the development of immunity (e.g., HIV immunity) in a subject after mucosal administration (e.g., mucosal vaccination) with a composition comprising a nanoemulsion and an immunogenic protein from HIV (e.g., recombinant gp120) generated and characterized during development of the present invention (See Examples 17-23). Nanoemulsion (NE), a surface-active antimicrobial material, was mixed with recombinant gp120 from either BaL or SF162 serotypes, resulting in an immunogenic composition comprising NE and recombinant gp120 that is stable at room temperature (e.g., in some embodiments, for more than 2 weeks, more preferably more than 3 weeks, even more preferably more than 4 weeks, and most preferably for more than 5 weeks) and that can be used to induce an immune response against HIV in a subject (e.g., that can be used either alone or as an adjuvant for inducing an anti-HIV immune response).

Mucosal administration of a composition comprising NE and an HIV immunogen (e.g., recombinant gp120) to a subject resulted in high-titer mucosal and systemic antibody responses and generated a Th1 type cellular immune response (See, e.g., Examples 17, 18, and 21). Further, antibodies generated against one serotype of gp120 cross-reacted with other gp120 serotypes (See, e.g., Example 19). Moreover, mice immunized intranasally with a composition comprising NE and recombinant gp120 generated mucosally secreted, anti-gp120 specific IgA antibodies that were detectable in both bronchial as well as vaginal mucosal surfaces (See Example 20). Thus, mice administered a composition of the present invention generated a mucosal immune response to HIV. The immune response generated in mice administered a composition comprising a NE and recombinant gp120 was also capable of neutralizing HIV (See Example 22).

Thus, in some embodiments, the present invention provides that administration (e.g., mucosal administration) of a composition comprising NE and an HIV immunogen (e.g., recombinant gp120) is sufficient to induce a protective immune response against HIV in a subject (e.g., protective immunity (e.g., mucosal and systemic immunity)). In some embodiments, a subsequent administration (e.g., one or more boost administrations subsequent to a primary administration) to a subject provides the induction of an enhanced immune response to HIV in the subject. Thus, the present invention demonstrates that administration of a composition comprising NE and an HIV immunogen (e.g., recombinant gp120) to a subject provides protective immunity against AIDS.

Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, combining a NE and an HIV immunogen (e.g., recombinant gp120) from one or more serotypes of HIV stabilizes the HIV immunogen (e.g., recombinant gp120) and provides the proper substance for generation of an immune response. In other embodiments, because NE formulations are known to penetrate the mucosa through pores, they may carry immunogenic proteins to the submucosal location of dendritic cells (e.g., thereby initiating and/or stimulating an immune response).

Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, NE treatment (e.g., neutralization of HIV with a NE of the present invention) preserves important viral neutralizing epitopes (e.g., recognizable by a subject's immune system), stabilizing their hydrophobic and hydrophilic components in the oil and

water interface of the emulsion (e.g., thereby providing one or more immunogens (e.g., stabilized antigens) against which a subject can mount an immune response). In other embodiments, because NE formulations are known to penetrate the mucosa through pores, they may carry viral proteins to the submucosal location of dendritic cells (e.g., thereby initiating and/or stimulating an immune response).

Dendritic cells avidly phagocytose NE oil droplets and this could provide a means to internalize immunogenic proteins for antigen presentation. While other vaccines rely on inflammatory toxins or other immune stimuli for adjuvant activity (See, e.g., Holmgren and Czerkinsky, *Nature Med.* 2005, 11; 45-53), NEs have not been shown to be inflammatory when placed on the skin or mucous membranes in studies on animals and in humans. Thus, although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, a composition comprising a NE of the present invention (e.g., a composition comprising NE and recombinant HIV proteins (e.g., gp120) from one or more serotypes of HIV may act as a "physical" adjuvant (e.g., that transports and/or presents HIV proteins (e.g., gp120) to the immune system. In some preferred embodiments, mucosal administration of a composition of the present invention generates mucosal as well as systemic immunity (e.g., signs of mucosal immunity (e.g., generation of IgA antibody titers).

Both cellular and humoral immunity play a role in protection against HIV and both were induced with the NE formulations (See, e.g., Examples 18-22). Thus, in some embodiments, administration (e.g., mucosal administration) of a composition of the present invention to a subject results in the induction of both humoral (e.g., development of specific antibodies) and cellular (e.g., cytotoxic T lymphocyte) immune responses (e.g., against HIV proteins (gp120)). In some preferred embodiments, a composition of the present invention (e.g., a composition comprising a NE and recombinant gp120 from one or more serotypes of HIV) is used as a AIDS vaccine.

Furthermore, in preferred embodiments, a composition of the present invention induces (e.g., when administered to a subject) both systemic and mucosal immunity. Thus, in some preferred embodiments, administration of a composition of the present invention to a subject results in protection against an exposure (e.g., a mucosal exposure) to HIV. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action,

mucosal administration (e.g., vaccination) provides protection against HIV infection (e.g., that initiates at a mucosal surface). Although it has heretofore proven difficult to stimulate secretory IgA responses and protection against pathogens that invade at mucosal surfaces (See, e.g., Mestecky et al, Mucosal Immunology. 3ed edn. (Academic Press, San Diego, 2005)), the present invention provides compositions and methods for stimulating mucosal immunity (e.g., a protective IgA response) from a pathogen in a subject.

In some embodiments, the present invention provides a composition (e.g., a composition comprising a NE and immunogenic protein antigens from HIV (e.g., gp120) to serve as a mucosal vaccine. This material can easily be produced with NE and HIV protein (e.g., viral-derived gp120, live-virus-vector-derived gp120 and gp160, recombinant mammalian gp120, recombinant denatured antigens, small peptide segments of gp120 and gp41, V3 loop peptides (See, e.g., Example 17)), and induces both mucosal and systemic immunity (See, e.g., Examples 18-22). The ability to produce this formulation rapidly and administer it via mucosal (e.g., nasal or vaginal) instillation provides a vaccine that can be used in large-scale administrations (e.g., to a population of a town, village, city, state or country).

In some preferred embodiments, the present invention provides a composition for generating an immune response comprising a NE and an immunogen (e.g., a purified, isolated or synthetic HIV protein or derivative, variant, or analogue thereof; or, one or more serotypes of HIV inactivated by the nanoemulsion). When administered to a subject, a composition of the present invention stimulates an immune response against the immunogen within the subject. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, generation of an immune response (e.g., resulting from administration of a composition comprising a nanoemulsion and an immunogen) provides total or partial immunity to the subject (e.g., from signs, symptoms or conditions of a disease (e.g., AIDS)). Without being bound to any specific theory, protection and/or immunity from disease (e.g., the ability of a subject's immune system to prevent or attenuate (e.g., suppress) a sign, symptom or condition of disease) after exposure to an immunogenic composition of the present invention is due to adaptive (e.g., acquired) immune responses (e.g., immune responses mediated by B and T cells following exposure to a NE comprising an immunogen of the present invention (e.g., immune responses that exhibit increased specificity and reactivity towards HIV). Thus, in some embodiments, the

compositions and methods of the present invention are used prophylactically or therapeutically to prevent or attenuate a sign, symptom or condition associated with AIDS.

In some embodiments, a NE comprising an immunogen (e.g., a recombinant HIV protein) is administered alone. In some embodiments, a composition comprising a NE and an immunogen (e.g., a recombinant HIV protein) comprises one or more other agents (e.g., a pharmaceutically acceptable carrier, adjuvant, excipient, and the like). In some embodiments, a composition for stimulating an immune response of the present invention is administered in a manner to induce a humoral immune response. In some embodiments, a composition for stimulating an immune response of the present invention is administered in a manner to induce a cellular (e.g., cytotoxic T lymphocyte) immune response, rather than a humoral response. In some embodiments, a composition comprising a NE and an immunogen of the present invention induces both a cellular and humoral immune response.

B. Pathogens

The present invention is not limited to the use of any one specific type of pathogen. Indeed, compositions (e.g., comprising a NE and an immunogen) useful for generating an immune response (e.g., for use as a vaccine) to a variety of pathogens are within the scope of the present invention. Accordingly, in some embodiments, the present invention provides compositions for generating an immune response to bacterial pathogens (e.g., in vegetative or spore forms) including, but not limited to, *Bacillus cereus*, *Bacillus circulans* and *Bacillus megaterium*, *Bacillus anthracis*, bacteria of the genus *Brucella*, *Vibrio cholera*, *Coxiella burnetii*, *Francisella tularensis*, *Chlamydia psittaci*, *Ricinus communis*, *Rickettsia prowazekii*, bacteria of the genus *Salmonella* (e.g., *S. typhi*), bacteria of the genus *Shigella*, *Cryptosporidium parvum*, *Burkholderia pseudomallei*, *Clostridium perfringens*, *Clostridium botulinum*, *Vibrio cholerae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria gonorrhoea*, *Haemophilus influenzae*, *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*). In other embodiments, the present invention provides compositions for generating an immune response to viral pathogens including, but not limited to, influenza A virus, avian influenza virus, H5N1 influenza virus, West Nile virus, SARS virus, Marburg virus, Arenaviruses, Nipah virus, alphaviruses, filoviruses, herpes simplex virus I, herpes simplex virus II, sendai, sindbis, vaccinia, parvovirus, human

immunodeficiency virus, hepatitis B virus, hepatitis C virus, hepatitis A virus, cytomegalovirus, human papilloma virus, picornavirus, hantavirus, junin virus, and ebola virus). In still further embodiments, the present invention provides compositions for generating an immune response to fungal pathogens, including, but not limited to, *Candida albicans* and *parapsilosis*, *Aspergillus fumigatus* and *niger*, *Fusarium spp*, *Trichophyton spp*.

Bacteria for use in formulating a composition for generating an immune response of the present invention can be obtained from commercial sources, including, but not limited to, American Type Culture Collection (ATCC). In some embodiments, bacteria are passed in animals prior to being mixed with nanoemulsions in order to enhance their pathogenicity for each specific animal host for 5-10 passages (Sinai *et al.*, J. Infect. Dis., 141:193 (1980)). In some embodiments, the bacteria then are then isolated from the host animals, expanded in culture and stored at -80°C. Just before use, the bacteria are thawed and grown on an appropriate solid bacterial culture medium overnight. The next day, the bacteria are collected from the agar plate and suspended in a suitable liquid solution (*e.g.*, Brain Heart Infusion (BHI) broth). The concentration of bacteria is adjusted so that the bacteria count is approximately 1.5×10^8 colony forming units per ml (CFU/ml), based on the McFarland standard for bactericidal testing (Hendrichson and Krenz, 1991).

Viruses for use in formulating a composition for generating an immune response of the present invention can be obtained from commercial sources, including, but not limited to, ATCC. In some embodiments, viruses are passed in the prospective animal model for 5-10 times to enhance pathogenicity for each specific animal (Ginsberg and Johnson, Infect. Immun., 13:1221 (1976)). In some embodiments, the virus is collected and propagated in tissue culture and then purified using density gradient concentration and ultracentrifugation (Garlinghouse *et al.*, Lab Anim Sci., 37:437 (1987); and Mahy, Br. Med. Bull., 41:50 (1985)). The Plaque Forming Units (PFU) are calculated in the appropriate tissue culture cells.

Lethal dose and/or infectious dose for each pathogen can be calculated using any suitable method, including, but not limited to, by administering different doses of the pathogens to the animals by the infective route and identifying the doses which result in the expected result of either animal sickness or death based on previous publications (Fortier *et al.*, Infect Immun., 59:2922 (1991); Jacoby, Exp Gerontol., 29:89 (1994); and Salit *et al.*, Can J Microbiol., 30:1022 (1984)).

C. Nanoemulsions

The nanoemulsion vaccine compositions of the present invention are not limited to any particular nanoemulsion. Any number of suitable nanoemulsion compositions may be utilized in the vaccine compositions of the present invention, including, but not limited to, those disclosed in Hamouda *et al.*, J. Infect Dis., 180:1939 (1999); Hamouda and Baker, J. Appl. Microbiol., 89:397 (2000); and Donovan *et al.*, Antivir. Chem. Chemother., 11:41 (2000), as well as those shown in Tables 1 and 2 and Figures 4 and 9. Preferred nanoemulsions of the present invention are those that are effective in killing or inactivating pathogens and that are non-toxic to animals. Accordingly, preferred emulsion formulations utilize non-toxic solvents, such as ethanol, and achieve more effective killing at lower concentrations of emulsion. In preferred embodiments, nanoemulsions utilized in the methods of the present invention are stable, and do not decompose even after long storage periods (*e.g.*, one or more years). Additionally, preferred emulsions maintain stability even after exposure to high temperature and freezing. This is especially useful if they are to be applied in extreme conditions (*e.g.*, on a battlefield). In some embodiments, one of the nanoemulsions described in Table 1 and or Figures 4 or 9 is utilized.

In some preferred embodiments, the emulsions comprise (i) an aqueous phase; (ii) an oil phase; and at least one additional compound. In some embodiments of the present invention, these additional compounds are admixed into either the aqueous or oil phases of the composition. In other embodiments, these additional compounds are admixed into a composition of previously emulsified oil and aqueous phases. In certain of these embodiments, one or more additional compounds are admixed into an existing emulsion composition immediately prior to its use. In other embodiments, one or more additional compounds are admixed into an existing emulsion composition prior to the compositions immediate use.

Additional compounds suitable for use in the compositions of the present invention include but are not limited to one or more, organic, and more particularly, organic phosphate based solvents, surfactants and detergents, quaternary ammonium containing compounds, cationic halogen containing compounds, germination enhancers, interaction enhancers, and pharmaceutically acceptable compounds. Certain exemplary embodiments of the various compounds contemplated for use in the compositions of the present invention are presented below.

Table 1 Nanoemulsion Formulations		
Name	Oil Phase Formula	Water to Oil Phase Ratio (Vol/Vol)
X8P	1 vol. Tri(N-butyl)phosphate	4:1
	1 vol. TRITON X-100	
	8 vol. Soybean oil	
NN	86.5 g Glycerol monooleate	3:1
	60.1 ml Nonoxynol-9	
	24.2 g GENEROL 122	
	3.27 g Cetylpyridinium chloride	
	554 g Soybean oil	
W ₈₀ 8P	86.5 g Glycerol monooleate	3.2:1
	21.2 g Polysorbate 60	
	24.2 g GENEROL 122	
	3.27 g Cetylpyddinium chloride	
	4 ml Peppermint oil	
	554 g Soybean oil	
SS	86.5 g Glycerol monooleate	3.2:1
	21.2 g Polysorbate 60	(1% bismuth in water)
	24.2 g GENEROL 122	
	3.27 g Cetylpyridinium chloride	
	554 g Soybean oil	
Table 2 Nanoemulsion Formulations		
Nanoemulsion	Composition	
X8P	8% TRITON X-100; 8% Tributyl phosphate; 64% Soybean oil; 20% Water	
W ₂₀ 5EC	5% TWEEN 20; 8% Ethanol; 1% Cetylpyridinium Chloride; 64% Soybean oil; 22% Water	
EC	1% Cetylpyridinium Chloride; 8% Ethanol; 64% Soybean oil; 27% Water	

Y3EC	3% TYLOXAPOL; 1% Cetylpyridinium Chloride; 8% Ethanol; 64% Soybean oil; 24% Water
X4E	4% TRITON X-100; 8% Ethanol; 64% Soybean oil; 24% Water

Some embodiments of the present invention employ an oil phase containing ethanol. For example, in some embodiments, the emulsions of the present invention contain (i) an aqueous phase and (ii) an oil phase containing ethanol as the organic solvent and optionally a germination enhancer, and (iii) TYLOXAPOL as the surfactant (preferably 2-5%, more preferably 3%). This formulation is highly efficacious against microbes and is also non-irritating and non-toxic to mammalian users (and can thus be contacted with mucosal membranes).

In some other embodiments, the emulsions of the present invention comprise a first emulsion emulsified within a second emulsion, wherein (a) the first emulsion comprises (i) an aqueous phase; and (ii) an oil phase comprising an oil and an organic solvent; and (iii) a surfactant; and (b) the second emulsion comprises (i) an aqueous phase; and (ii) an oil phase comprising an oil and a cationic containing compound; and (iii) a surfactant.

The following description provides a number of exemplary emulsions including formulations for compositions X8P and X8W₆₀PC. X8P comprises a water-in oil nanoemulsion, in which the oil phase was made from soybean oil, tri-n-butyl phosphate, and TRITON X-100 in 80% water. X8W₆₀PC comprises a mixture of equal volumes of X8P with W₈₀8P. W₈₀8P is a liposome-like compound made of glycerol monostearate, refined soya sterols (*e.g.*, GENEROL sterols), TWEEN 60, soybean oil, a cationic ion halogen-containing CPC and peppermint oil. The GENEROL family are a group of a polyethoxylated soya sterols (Henkel Corporation, Ambler, Pennsylvania). Emulsion formulations are given in Table 1 for certain embodiments of the present invention. These particular formulations may be found in U.S. Pat. Nos. 5,700,679 (NN); 5,618,840; 5,549,901 (W₈₀8P); and 5,547,677, herein incorporated by reference in their entireties.

The X8W₆₀PC emulsion is manufactured by first making the W₈₀8P emulsion and X8P emulsions separately. A mixture of these two emulsions is then re-emulsified to produce a fresh emulsion composition termed X8W₆₀PC. Methods of producing such emulsions are described in U.S. Pat. Nos. 5,103,497 and 4,895,452 (herein incorporated by

reference in their entirety). These compounds have broad-spectrum antimicrobial activity, and are able to inactivate vegetative bacteria through membrane disruption.

The compositions listed above are only exemplary and those of skill in the art will be able to alter the amounts of the components to arrive at a nanoemulsion composition suitable for the purposes of the present invention. Those skilled in the art will understand that the ratio of oil phase to water as well as the individual oil carrier, surfactant CPC and organic phosphate buffer, components of each composition may vary.

Although certain compositions comprising X8P have a water to oil ratio of 4:1, it is understood that the X8P may be formulated to have more or less of a water phase. For example, in some embodiments, there is 3, 4, 5, 6, 7, 8, 9, 10, or more parts of the water phase to each part of the oil phase. The same holds true for the W808P formulation. Similarly, the ratio of Tri(N-butyl)phosphate:TRITON X-100:soybean oil also may be varied.

Although Table 1 lists specific amounts of glycerol monooleate, polysorbate 60, GENEROL 122, cetylpyridinium chloride, and carrier oil for W808P, these are merely exemplary. An emulsion that has the properties of W808P may be formulated that has different concentrations of each of these components or indeed different components that will fulfill the same function. For example, the emulsion may have between about 80 to about 100g of glycerol monooleate in the initial oil phase. In other embodiments, the emulsion may have between about 15 to about 30 g polysorbate 60 in the initial oil phase. In yet another embodiment the composition may comprise between about 20 to about 30 g of a GENEROL sterol, in the initial oil phase.

The nanoemulsions structure of the certain embodiments of the emulsions of the present invention may play a role in their biocidal activity as well as contributing to the non-toxicity of these emulsions. For example, the active component in X8P, TRITON-X100 shows less biocidal activity against virus at concentrations equivalent to 11% X8P. Adding the oil phase to the detergent and solvent markedly reduces the toxicity of these agents in tissue culture at the same concentrations. While not being bound to any theory (an understanding of the mechanism is not necessary to practice the present invention, and the present invention is not limited to any particular mechanism), it is suggested that the nanoemulsion enhances the interaction of its components with the pathogens thereby facilitating the inactivation of the pathogen and reducing the toxicity of

the individual components. It should be noted that when all the components of X8P are combined in one composition but are not in a nanoemulsion structure, the mixture is not as effective as an antimicrobial as when the components are in a nanoemulsion structure.

Numerous additional embodiments presented in classes of formulations with like compositions are presented below. The effect of a number of these compositions as antipathogenic materials is provided in Figure 9. The following compositions recite various ratios and mixtures of active components. One skilled in the art will appreciate that the below recited formulation are exemplary and that additional formulations comprising similar percent ranges of the recited components are within the scope of the present invention.

In certain embodiments of the present invention, the inventive formulation comprise from about 3 to 8 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of cetylpyridinium chloride (CPC), about 60 to 70 vol. % oil (*e.g.*, soybean oil), about 15 to 25 vol. % of aqueous phase (*e.g.*, DiH_2O or PBS), and in some formulations less than about 1 vol. % of 1N NaOH. Some of these embodiments comprise PBS. It is contemplated that the addition of 1N NaOH and/or PBS in some of these embodiments, allows the user to advantageously control the pH of the formulations, such that pH ranges from about 4.0 to about 10.0, and more preferably from about 7.1 to 8.5 are achieved. For example, one embodiment of the present invention comprises about 3 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 24 vol. % of DiH_2O (designated herein as Y3EC). Another similar embodiment comprises about 3.5 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, and about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 23.5 vol. % of DiH_2O (designated herein as Y3.5EC). Yet another embodiment comprises about 3 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 0.067 vol. % of 1N NaOH, such that the pH of the formulation is about 7.1, about 64 vol. % of soybean oil, and about 23.93 vol. % of DiH_2O (designated herein as Y3EC pH 7.1). Still another embodiment comprises about 3 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 0.67 vol. % of 1N NaOH, such that the pH of the formulation is about 8.5, and about 64 vol. % of soybean oil, and about 23.33 vol. % of DiH_2O (designated herein as Y3EC pH 8.5). Another similar embodiment comprises about 4% TYLOXAPOL, about 8 vol. % ethanol, about 1% CPC, and about 64 vol. % of soybean oil, and about 23 vol. % of DiH_2O (designated herein as

Y4EC). In still another embodiment the formulation comprises about 8% TYLOXAPOL, about 8% ethanol, about 1 vol. % of CPC, and about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O (designated herein as Y8EC). A further embodiment comprises about 8 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 19 vol. % of 1x PBS (designated herein as Y8EC PBS).

In some embodiments of the present invention, the inventive formulations comprise about 8 vol. % of ethanol, and about 1 vol. % of CPC, and about 64 vol. % of oil (*e.g.*, soybean oil), and about 27 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS) (designated herein as EC).

In the present invention, some embodiments comprise from about 8 vol. % of sodium dodecyl sulfate (SDS), about 8 vol. % of tributyl phosphate (TBP), and about 64 vol. % of oil (*e.g.*, soybean oil), and about 20 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS) (designated herein as S8P).

In certain embodiments of the present invention, the inventive formulation comprise from about 1 to 2 vol. % of TRITON X-100, from about 1 to 2 vol. % of TYLOXAPOL, from about 7 to 8 vol. % of ethanol, about 1 vol. % of cetylpyridinium chloride (CPC), about 64 to 57.6 vol. % of oil (*e.g.*, soybean oil), and about 23 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS). Additionally, some of these formulations further comprise about 5 mM of L-alanine/Inosine, and about 10 mM ammonium chloride. Some of these formulations comprise PBS. It is contemplated that the addition of PBS in some of these embodiments, allows the user to advantageously control the pH of the formulations. For example, one embodiment of the present invention comprises about 2 vol. % of TRITON X-100, about 2 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % CPC, about 64 vol. % of soybean oil, and about 23 vol. % of aqueous phase DiH₂O. In another embodiment the formulation comprises about 1.8 vol. % of TRITON X-100, about 1.8 vol. % of TYLOXAPOL, about 7.2 vol. % of ethanol, about 0.9 vol. % of CPC, about 5 mM L-alanine/Inosine, and about 10 mM ammonium chloride, about 57.6 vol. % of soybean oil, and the remainder of 1x PBS (designated herein as 90% X2Y2EC/GE).

In alternative embodiments of the present invention, the formulations comprise from about 5 vol. % of TWEEN 80, from about 8 vol. % of ethanol, from about 1 vol. % of CPC, about 64 vol. % of oil (*e.g.*, soybean oil), and about 22 vol. % of DiH₂O (designated herein as W₈₀5EC).

In still other embodiments of the present invention, the formulations comprise from about 5 vol. % of TWEEN 20, from about 8 vol. % of ethanol, from about 1 vol. % of CPC, about 64 vol. % of oil (*e.g.*, soybean oil), and about 22 vol. % of DiH₂O (designated herein as W₂₀5EC).

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In still other embodiments of the present invention, the formulations comprise from about 2 to 8 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 60 to 70 vol. % of oil (*e.g.*, soybean, or olive oil), and about 15 to 25 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS). For example, the present invention contemplates formulations comprising about 2 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 26 vol. % of DiH₂O (designated herein as X2E). In other similar embodiments, the formulations comprise about 3 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 25 vol. % of DiH₂O (designated herein as X3E). In still further embodiments, the formulations comprise about 4 vol. % TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 24 vol. % of DiH₂O (designated herein as X4E). In yet other embodiments, the formulations comprise about 5 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 23 vol. % of DiH₂O (designated herein as X5E). Another embodiment of the present invention comprises about 6 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 22 vol. % of DiH₂O (designated herein as X6E). In still further embodiments of the present invention, the formulations comprise about 8 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as X8E). In still further embodiments of the present invention, the formulations comprise about 8 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of olive oil, and about 20 vol. % of DiH₂O (designated herein as X8E O). In yet another embodiment comprises 8 vol. % of TRITON X-100, about 8 vol. % ethanol, about 1 vol. % CPC, about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O (designated herein as X8EC).

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In alternative embodiments of the present invention, the formulations comprise from about 1 to 2 vol. % of TRITON X-100, from about 1 to 2 vol. % of TYLOXAPOL, from about 6 to 8 vol. % TBP, from about 0.5 to 1.0 vol. % of CPC, from about 60 to 70 vol. % of oil (*e.g.*, soybean), and about 1 to 35 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS).

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Additionally, certain of these formulations may comprise from about 1 to 5 vol. % of trypticase soy broth, from about 0.5 to 1.5 vol. % of yeast extract, about 5 mM L-alanine/Inosine, about 10 mM ammonium chloride, and from about 20-40 vol. % of liquid baby formula. In some of the embodiments comprising liquid baby formula, the formula comprises a casein hydrolysate (*e.g.*, Neutramigen, or Progestimil, and the like). In some of these embodiments, the inventive formulations further comprise from about 0.1 to 1.0 vol. % of sodium thiosulfate, and from about 0.1 to 1.0 vol. % of sodium citrate. Other similar embodiments comprising these basic components employ phosphate buffered saline (PBS) as the aqueous phase. For example, one embodiment comprises about 2 vol. % of TRITON X-100, about 2 vol. % TYLOXAPOL, about 8 vol. % TBP, about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 23 vol. % of DiH₂O (designated herein as X2Y2EC). In still other embodiments, the inventive formulation comprises about 2 vol. % of TRITON X-100, about 2 vol. % TYLOXAPOL, about 8 vol. % TBP, about 1 vol. % of CPC, about 0.9 vol. % of sodium thiosulfate, about 0.1 vol. % of sodium citrate, about 64 vol. % of soybean oil, and about 22 vol. % of DiH₂O (designated herein as X2Y2PC STS1). In another similar embodiment, the formulations comprise about 1.7 vol. % TRITON X-100, about 1.7 vol. % TYLOXAPOL, about 6.8 vol. % TBP, about 0.85% CPC, about 29.2% NEUTRAMIGEN, about 54.4 vol. % of soybean oil, and about 4.9 vol. % of DiH₂O (designated herein as 85% X2Y2PC/baby). In yet another embodiment of the present invention, the formulations comprise about 1.8 vol. % of TRITON X-100, about 1.8 vol. % of TYLOXAPOL, about 7.2 vol. % of TBP, about 0.9 vol. % of CPC, about 5mM L-alanine/Inosine, about 10mM ammonium chloride, about 57.6 vol. % of soybean oil, and the remainder vol. % of 0.1x PBS (designated herein as 90% X2Y2 PC/GE). In still another embodiment, the formulations comprise about 1.8 vol. % of TRITON X-100, about 1.8 vol. % of TYLOXAPOL, about 7.2 vol. % TBP, about 0.9 vol. % of CPC, and about 3 vol. % trypticase soy broth, about 57.6 vol. % of soybean oil, and about 27.7 vol. % of DiH₂O (designated herein as 90% X2Y2PC/TSB). In another embodiment of the present invention, the formulations comprise about 1.8 vol. % TRITON X-100, about 1.8 vol. % TYLOXAPOL, about 7.2 vol. % TBP, about 0.9 vol. % CPC, about 1 vol. % yeast extract, about 57.6 vol. % of soybean oil, and about 29.7 vol. % of DiH₂O (designated herein as 90% X2Y2PC/YE).

In some embodiments of the present invention, the inventive formulations comprise

about 3 vol. % of TYLOXAPOL, about 8 vol. % of TBP, and about 1 vol. % of CPC, about 60 to 70 vol. % of oil (*e.g.*, soybean or olive oil), and about 15 to 30 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS). In a particular embodiment of the present invention, the inventive formulations comprise about 3 vol. % of TYLOXAPOL, about 8 vol. % of TBP, and about 1 vol. % of CPC, about 64 vol. % of soybean, and about 24 vol. % of DiH₂O (designated herein as Y3PC).

In some embodiments of the present invention, the inventive formulations comprise from about 4 to 8 vol. % of TRITON X-100, from about 5 to 8 vol. % of TBP, about 30 to 70 vol. % of oil (*e.g.*, soybean or olive oil), and about 0 to 30 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS). Additionally, certain of these embodiments further comprise about 1 vol. % of CPC, about 1 vol. % of benzalkonium chloride, about 1 vol. % cetylridinium bromide, about 1 vol. % cetyldimethyletylammonium bromide, 500 μ M EDTA, about 10 mM ammonium chloride, about 5 mM Inosine, and about 5 mM L-alanine. For example, in certain of these embodiments, the inventive formulations comprise about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as X8P). In another embodiment of the present invention, the inventive formulations comprise about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1% of CPC, about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O (designated herein as X8PC). In still another embodiment, the formulations comprise about 8 vol. % TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of CPC, about 50 vol. % of soybean oil, and about 33 vol. % of DiH₂O (designated herein as ATB-X1001). In yet another embodiment, the formulations comprise about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 2 vol. % of CPC, about 50 vol. % of soybean oil, and about 32 vol. % of DiH₂O (designated herein as ATB-X002). Another embodiment of the present invention comprises about 4 vol. % TRITON X-100, about 4 vol. % of TBP, about 0.5 vol. % of CPC, about 32 vol. % of soybean oil, and about 59.5 vol. % of DiH₂O (designated herein as 50% X8PC). Still another related embodiment comprises about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 0.5 vol. % CPC, about 64 vol. % of soybean oil, and about 19.5 vol. % of DiH₂O (designated herein as X8PC_{1/2}). In some embodiments of the present invention, the inventive formulations comprise about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 2 vol. % of CPC, about 64 vol. % of soybean oil, and about 18

vol. % of DiH₂O (designated herein as X8PC2). In other embodiments, the inventive formulations comprise about 8 vol. % of TRITON X-100, about 8% of TBP, about 1% of benzalkonium chloride, about 50 vol. % of soybean oil, and about 33 vol. % of DiH₂O (designated herein as X8P BC). In an alternative embodiment of the present invention, the formulation comprise about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of cetylyridinium bromide, about 50 vol. % of soybean oil, and about 33 vol. % of DiH₂O (designated herein as X8P CPB). In another exemplary embodiment of the present invention, the formulations comprise about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of cetyldimethyletylammonium bromide, about 50 vol. % of soybean oil, and about 33 vol. % of DiH₂O (designated herein as X8P CTAB). In still further embodiments, the present invention comprises about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of CPC, about 500 μM EDTA, about 64 vol. % of soybean oil, and about 15.8 vol. % DiH₂O (designated herein as X8PC EDTA). Additional similar embodiments comprise 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of CPC, about 10 mM ammonium chloride, about 5mM Inosine, about 5mM L-alanine, about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O or PBS (designated herein as X8PC GE_{1x}). In another embodiment of the present invention, the inventive formulations further comprise about 5 vol. % of TRITON X-100, about 5% of TBP, about 1 vol. % of CPC, about 40 vol. % of soybean oil, and about 49 vol. % of DiH₂O (designated herein as X5P₅C).

In some embodiments of the present invention, the inventive formulations comprise about 2 vol. % TRITON X-100, about 6 vol. % TYLOXAPOL, about 8 vol. % ethanol, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as X2Y6E).

In an additional embodiment of the present invention, the formulations comprise about 8 vol. % of TRITON X-100, and about 8 vol. % of glycerol, about 60 to 70 vol. % of oil (*e.g.*, soybean or olive oil), and about 15 to 25 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS). Certain related embodiments further comprise about 1 vol. % L-ascorbic acid. For example, one particular embodiment comprises about 8 vol. % of TRITON X-100, about 8 vol. % of glycerol, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as X8G). In still another embodiment, the inventive formulations

comprise about 8 vol. % of TRITON X-100, about 8 vol. % of glycerol, about 1 vol. % of L-ascorbic acid, about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O (designated herein as X8GV_C).

In still further embodiments, the inventive formulations comprise about 8 vol. % of TRITON X-100, from about 0.5 to 0.8 vol. % of TWEEN 60, from about 0.5 to 2.0 vol. % of CPC, about 8 vol. % of TBP, about 60 to 70 vol. % of oil (*e.g.*, soybean or olive oil), and about 15 to 25 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS). For example, in one particular embodiment the formulations comprise about 8 vol. % of TRITON X-100, about 0.70 vol. % of TWEEN 60, about 1 vol. % of CPC, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 18.3 vol. % of DiH₂O (designated herein as X8W60PC₁).

Another related embodiment comprises about 8 vol. % of TRITON X-100, about 0.71 vol. % of TWEEN 60, about 1 vol. % of CPC, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 18.29 vol. % of DiH₂O (designated herein as W60_{0.7}X8PC). In yet other embodiments, the inventive formulations comprise from about 8 vol. % of TRITON X-100, about 0.7 vol. % of TWEEN 60, about 0.5 vol. % of CPC, about 8 vol. % of TBP, about 64 to 70 vol. % of soybean oil, and about 18.8 vol. % of DiH₂O (designated herein as X8W60PC₂). In still other embodiments, the present invention comprises about 8 vol. % of TRITON X-100, about 0.71 vol. % of TWEEN 60, about 2 vol. % of CPC, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 17.3 vol. % of DiH₂O. In another embodiment of the present invention, the formulations comprise about 0.71 vol. % of TWEEN 60, about 1 vol. % of CPC, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 25.29 vol. % of DiH₂O (designated herein as W60_{0.7}PC).

In another embodiment of the present invention, the inventive formulations comprise about 2 vol. % of dioctyl sulfosuccinate, either about 8 vol. % of glycerol, or about 8 vol. % of TBP, in addition to, about 60 to 70 vol. % of oil (*e.g.*, soybean or olive oil), and about 20 to 30 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS). For example, one embodiment of the present invention comprises about 2 vol. % of dioctyl sulfosuccinate, about 8 vol. % of glycerol, about 64 vol. % of soybean oil, and about 26 vol. % of DiH₂O (designated herein as D2G). In another related embodiment, the inventive formulations comprise about 2 vol. % of dioctyl sulfosuccinate, and about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 26 vol. % of DiH₂O (designated herein as D2P).

In still other embodiments of the present invention, the inventive formulations comprise about 8 to 10 vol. % of glycerol, and about 1 to 10 vol. % of CPC, about 50 to 70 vol. % of oil (*e.g.*, soybean or olive oil), and about 15 to 30 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS). Additionally, in certain of these embodiments, the compositions further comprise about 1 vol. % of L-ascorbic acid. For example, one particular embodiment comprises about 8 vol. % of glycerol, about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 27 vol. % of DiH₂O (designated herein as GC). An additional related embodiment comprises about 10 vol. % of glycerol, about 10 vol. % of CPC, about 60 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as GC10). In still another embodiment of the present invention, the inventive formulations comprise about 10 vol. % of glycerol, about 1 vol. % of CPC, about 1 vol. % of L-ascorbic acid, about 64 vol. % of soybean or oil, and about 24 vol. % of DiH₂O (designated herein as GCV_C).

In some embodiments of the present invention, the inventive formulations comprise about 8 to 10 vol. % of glycerol, about 8 to 10 vol. % of SDS, about 50 to 70 vol. % of oil (*e.g.*, soybean or olive oil), and about 15 to 30 vol. % of aqueous phase (*e.g.*, DiH₂O or PBS). Additionally, in certain of these embodiments, the compositions further comprise about 1 vol. % of lecithin, and about 1 vol. % of p-Hydroxybenzoic acid methyl ester. Exemplary embodiments of such formulations comprise about 8 vol. % SDS, 8 vol. % of glycerol, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as S8G). A related formulation comprises about 8 vol. % of glycerol, about 8 vol. % of SDS, about 1 vol. % of lecithin, about 1 vol. % of p-Hydroxybenzoic acid methyl ester, about 64 vol. % of soybean oil, and about 18 vol. % of DiH₂O (designated herein as S8GL1B1).

In yet another embodiment of the present invention, the inventive formulations comprise about 4 vol. % of TWEEN 80, about 4 vol. % of TYLOXAPOL, about 1 vol. % of CPC, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O (designated herein as W₈₀4Y4EC).

In some embodiments of the present invention, the inventive formulations comprise about 0.01 vol. % of CPC, about 0.08 vol. % of TYLOXAPOL, about 10 vol. % of ethanol, about 70 vol. % of soybean oil, and about 19.91 vol. % of DiH₂O (designated herein as Y.08EC.01).

In yet another embodiment of the present invention, the inventive formulations

comprise about 8 vol. % of sodium lauryl sulfate, and about 8 vol. % of glycerol, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as SLS8G).

The specific formulations described above are simply examples to illustrate the variety of compositions that find use in the present invention. The present invention contemplates that many variations of the above formulation, as well as additional nanoemulsions, find use in the methods of the present invention. To determine if a candidate emulsion is suitable for use with the present invention, three criteria may be analyzed. Using the methods and standards described herein, candidate emulsions can be easily tested to determine if they are suitable. First, the desired ingredients are prepared using the methods described herein, to determine if an emulsion can be formed. If an emulsion cannot be formed, the candidate is rejected. For example, a candidate composition made of 4.5% sodium thiosulfate, 0.5% sodium citrate, 10% n-butanol, 64% soybean oil, and 21% DiH₂O did not form an emulsion.

Second, in preferred embodiments, the candidate emulsion should form a stable emulsion. An emulsion is stable if it remains in emulsion form for a sufficient period to allow its intended use. For example, for emulsions that are to be stored, shipped, etc., it may be desired that the composition remain in emulsion form for months to years. Typical emulsions that are relatively unstable, will lose their form within a day. For example, a candidate composition made of 8% 1-butanol, 5% TWEEN 10, 1% CPC, 64% soybean oil, and 22% DiH₂O did not form a stable emulsion. The following candidate emulsions were shown to be stable using the methods described herein: 0.08% TRITON X-100, 0.08% Glycerol, 0.01% Cetylpyridinium Chloride, 99% Butter, and 0.83% diH₂O (designated herein as 1% X8GC Butter); 0.8% TRITON X-100, 0.8% Glycerol, 0.1% Cetylpyridinium Chloride, 6.4% Soybean Oil, 1.9% diH₂O, and 90% Butter (designated herein as 10% X8GC Butter); 2% W₂₀5EC, 1% Natrosol 250L NF, and 97% diH₂O (designated herein as 2% W₂₀5EC L GEL); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Ethanol, 64% 70 Viscosity Mineral Oil, and 22% diH₂O (designated herein as W₂₀5EC 70 Mineral Oil); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Ethanol, 64% 350 Viscosity Mineral Oil, and 22% diH₂O (designated herein as W₂₀5EC 350 Mineral Oil).

Third, the candidate emulsion should have efficacy for its intended use. For example, an anti-bacterial emulsion should kill or disable pathogens to a detectable level. As shown herein, certain emulsions of the present invention have efficacy against specific

microorganisms, but not against others. Using the methods described herein, one is capable of determining the suitability of a particular candidate emulsion against the desired microorganism. Generally, this involves exposing the microorganism to the emulsion for one or more time periods in a side-by-side experiment with the appropriate control samples (e.g., a negative control such as water) and determining if, and to what degree, the emulsion kills or disables the microorganism. For example, a candidate composition made of 1% ammonium chloride, 5% TWEEN 20, 8% ethanol, 64% soybean oil, and 22% diH₂O was shown not to be an effective emulsion. The following candidate emulsions were shown to be effective using the methods described herein: 5% TWEEN 20, 5% Cetylpyridinium Chloride, 10% Glycerol, 60% Soybean Oil, and 20% diH₂O (designated herein as W₂₀5GC5); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 10% Glycerol, 64% Soybean Oil, and 20% diH₂O (designated herein as W₂₀5GC); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Ethanol, 64% Olive Oil, and 22% diH₂O (designated herein as W₂₀5EC Olive Oil); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Ethanol, 64% Flaxseed Oil, and 22% diH₂O (designated herein as W₂₀5EC Flaxseed Oil); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Ethanol, 64% Corn Oil, and 22% diH₂O (designated herein as W₂₀5EC Corn Oil); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Ethanol, 64% Coconut Oil, and 22% diH₂O (designated herein as W₂₀5EC Coconut Oil); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Ethanol, 64% Cottonseed Oil, and 22% diH₂O (designated herein as W₂₀5EC Cottonseed Oil); 8% Dextrose, 5% TWEEN 10, 1% Cetylpyridinium Chloride, 64% Soybean Oil, and 22% diH₂O (designated herein as W₂₀5C Dextrose); 8% PEG 200, 5% TWEEN 10, 1% Cetylpyridinium Chloride, 64% Soybean Oil, and 22% diH₂O (designated herein as W₂₀5C PEG 200); 8% Methanol, 5% TWEEN 10, 1% Cetylpyridinium Chloride, 64% Soybean Oil, and 22% diH₂O (designated herein as W₂₀5C Methanol); 8% PEG 1000, 5% TWEEN 10, 1% Cetylpyridinium Chloride, 64% Soybean Oil, and 22% diH₂O (designated herein as W₂₀5C PEG 1000); 2% W₂₀5EC, 2% Natrosol 250H NF, and 96% diH₂O (designated herein as 2% W₂₀5EC Natrosol 2, also called 2% W₂₀5EC GEL); 2% W₂₀5EC, 1% Natrosol 250H NF, and 97% diH₂O (designated herein as 2% W₂₀5EC Natrosol 1); 2% W₂₀5EC, 3% Natrosol 250H NF, and 95% diH₂O (designated herein as 2% W₂₀5EC Natrosol 3); 2% W₂₀5EC, 0.5% Natrosol

250H NF, and 97.5% diH₂O (designated herein as 2% W₂₀5EC Natrosol 0.5); 2% W₂₀5EC, 2% Methocel A, and 96% diH₂O (designated herein as 2% W₂₀5EC Methocel A); 2% W₂₀5EC, 2% Methocel K, and 96% diH₂O (designated herein as 2% W₂₀5EC Methocel K); 2% Natrosol, 0.1% X8PC, 0.1x PBS, 5 mM L-alanine, 5 mM Inosine, 10 mM Ammonium Chloride, and diH₂O (designated herein as 0.1% X8PC/GE+2% Natrosol); 2% Natrosol, 0.8% TRITON X-100, 0.8% Tributyl Phosphate, 6.4% Soybean Oil, 0.1% Cetylpyridinium Chloride, 0.1x PBS, 5 mM L-alanine, 5 mM Inosine, 10 mM Ammonium Chloride, and diH₂O (designated herein as 10% X8PC/GE+2% Natrosol); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Ethanol, 64% Lard, and 22% diH₂O (designated herein as W₂₀5EC Lard); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Ethanol, 64% Mineral Oil, and 22% diH₂O (designated herein as W₂₀5EC Mineral Oil); 0.1% Cetylpyridinium Chloride, 2% Nerolidol, 5% TWEEN 20, 10% Ethanol, 64% Soybean Oil, and 18.9% diH₂O (designated herein as W₂₀5EC_{0.1}N); 0.1% Cetylpyridinium Chloride, 2% Farnesol, 5% TWEEN 20, 10% Ethanol, 64% Soybean Oil, and 18.9% diH₂O (designated herein as W₂₀5EC_{0.1}F); 0.1% Cetylpyridinium Chloride, 5% TWEEN 20, 10% Ethanol, 64% Soybean Oil, and 20.9% diH₂O (designated herein as W₂₀5EC_{0.1}); 10% Cetylpyridinium Chloride, 8% Tributyl Phosphate, 8% TRITON X-100, 54% Soybean Oil, and 20% diH₂O (designated herein as X8PC₁₀); 5% Cetylpyridinium Chloride, 8% TRITON X-100, 8% Tributyl Phosphate, 59% Soybean Oil, and 20% diH₂O (designated herein as X8PC₅); 0.02% Cetylpyridinium Chloride, 0.1% TWEEN 20, 10% Ethanol, 70% Soybean Oil, and 19.88% diH₂O (designated herein as W₂₀0.1EC_{0.02}); 1% Cetylpyridinium Chloride, 5% TWEEN 20, 8% Glycerol, 64% Mobil 1, and 22% diH₂O (designated herein as W₂₀5GC Mobil 1); 7.2% TRITON X-100, 7.2% Tributyl Phosphate, 0.9% Cetylpyridinium Chloride, 57.6% Soybean Oil, 0.1x PBS, 5 mM L-alanine, 5 mM Inosine, 10 mM Ammonium Chloride, and 25.87% diH₂O (designated herein as 90% X8PC/GE); 7.2% TRITON X-100, 7.2% Tributyl Phosphate, 0.9% Cetylpyridinium Chloride, 57.6% Soybean Oil, 1% EDTA, 5 mM L-alanine, 5 mM Inosine, 10 mM Ammonium Chloride, 0.1x PBS, and diH₂O (designated herein as 90% X8PC/GE EDTA); and 7.2% TRITON X-100, 7.2% Tributyl Phosphate, 0.9% Cetylpyridinium Chloride, 57.6% Soybean Oil, 1% Sodium Thiosulfate, 5 mM L-alanine, 5 mM Inosine, 10 mM

Ammonium Chloride, 0.1x PBS, and diH₂O (designated herein as 90% X8PC/GE STS).

1. Aqueous Phase

In some embodiments, the emulsion comprises an aqueous phase. In certain preferred embodiments, the emulsion comprises about 5 to 50, preferably 10 to 40, more preferably 15 to 30, vol. % aqueous phase, based on the total volume of the emulsion (although other concentrations are also contemplated). In preferred embodiments, the aqueous phase comprises water at a pH of about 4 to 10, preferably about 6 to 8. The water is preferably deionized (hereinafter "DiH₂O"). In some embodiments, the aqueous phase comprises phosphate buffered saline (PBS). In some preferred embodiments, the aqueous phase is sterile and pyrogen free.

2. Oil Phase

In some embodiments, the emulsion comprises an oil phase. In certain preferred embodiments, the oil phase (*e.g.*, carrier oil) of the emulsion of the present invention comprises 30-90, preferably 60-80, and more preferably 60-70, vol. % of oil, based on the total volume of the emulsion (although other concentrations are also contemplated). Suitable oils include, but are not limited to, soybean oil, avocado oil, squalene oil, squalene oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, flavor oils, water insoluble vitamins and mixtures thereof. In particularly preferred embodiments, soybean oil is used. In preferred embodiments of the present invention, the oil phase is preferably distributed throughout the aqueous phase as droplets having a mean particle size in the range from about 1-2 microns, more preferably from 0.2 to 0.8, and most preferably about 0.8 microns. In other embodiments, the aqueous phase can be distributed in the oil phase.

In some embodiments, the oil phase comprises 3-15, and preferably 5-10 vol. % of an organic solvent, based on the total volume of the emulsion. While the present invention is not limited to any particular mechanism, it is contemplated that the organic phosphate-based solvents employed in the emulsions serve to remove or disrupt the lipids in the membranes of the pathogens. Thus, any solvent that removes the sterols or phospholipids in the microbial membranes finds use in the methods of the present invention. Suitable organic solvents include, but are not limited to, organic phosphate based solvents or alcohols. In some preferred embodiments, non-toxic alcohols (*e.g.*, ethanol) are used as a

solvent. The oil phase, and any additional compounds provided in the oil phase, are preferably sterile and pyrogen free.

3. Surfactants and Detergents

In some embodiments, the emulsions further comprises a surfactant or detergent. In some preferred embodiments, the emulsion comprises from about 3 to 15 %, and preferably about 10 % of one or more surfactants or detergents (although other concentrations are also contemplated). While the present invention is not limited to any particular mechanism, it is contemplated that surfactants, when present in the emulsions, help to stabilize the emulsions. Both non-ionic (non-anionic) and ionic surfactants are contemplated. Additionally, surfactants from the BRIJ family of surfactants find use in the compositions of the present invention. The surfactant can be provided in either the aqueous or the oil phase. Surfactants suitable for use with the emulsions include a variety of anionic and nonionic surfactants, as well as other emulsifying compounds that are capable of promoting the formation of oil-in-water emulsions. In general, emulsifying compounds are relatively hydrophilic, and blends of emulsifying compounds can be used to achieve the necessary qualities. In some formulations, nonionic surfactants have advantages over ionic emulsifiers in that they are substantially more compatible with a broad pH range and often form more stable emulsions than do ionic (*e.g.*, soap-type) emulsifiers. Thus, in certain preferred embodiments, the compositions of the present invention comprise one or more non-ionic surfactants such as polysorbate surfactants (*e.g.*, polyoxyethylene ethers), polysorbate detergents, phoxypolyethoxyethanols, and the like. Examples of polysorbate detergents useful in the present invention include, but are not limited to, TWEEN 20, TWEEN 40, TWEEN 60, TWEEN 80, etc.

TWEEN 60 (polyoxyethylenesorbitan monostearate), together with TWEEN 20, TWEEN 40 and TWEEN 80, comprise polysorbates that are used as emulsifiers in a number of pharmaceutical compositions. In some embodiments of the present invention, these compounds are also used as co-components with adjuvants. TWEEN surfactants also appear to have virucidal effects on lipid-enveloped viruses (*See e.g.*, Eriksson *et al.*, Blood Coagulation and Fibrinolysis 5 (Suppl. 3):S37-S44 (1994)).

Examples of phoxypolyethoxyethanols, and polymers thereof, useful in the present invention include, but are not limited to, TRITON (*e.g.*, X-100, X-301, X-165, X-102, X-200), and TYLOXAPOL. TRITON X-100 is a strong non-ionic detergent and dispersing

agent widely used to extract lipids and proteins from biological structures. It also has virucidal effect against broad spectrum of enveloped viruses (*See e.g.*, Maha and Igarashi, Southeast Asian J. Trop. Med. Pub. Health 28:718 (1997); and Portocala *et al.*, Virologie 27:261 (1976)). Due to this anti-viral activity, it is employed to inactivate viral pathogens in fresh frozen human plasma (*See e.g.*, Horowitz *et al.*, Blood 79:826 (1992)).

The present invention is not limited to the surfactants disclosed herein. Additional surfactants and detergents useful in the compositions of the present invention may be ascertained from reference works (*e.g.*, including, but not limited to, McCutcheon's Volume 1: Emulsions and Detergents - North American Edition, 2000) and commercial sources.

4. Cationic Halogens Containing Compounds

In some embodiments, the emulsions further comprise a cationic halogen containing compound. In some preferred embodiments, the emulsion comprises from about 0.5 to 1.0 wt. % or more of a cationic halogen containing compound, based on the total weight of the emulsion (although other concentrations are also contemplated). In preferred embodiments, the cationic halogen-containing compound is preferably premixed with the oil phase; however, it should be understood that the cationic halogen-containing compound may be provided in combination with the emulsion composition in a distinct formulation. Suitable halogen containing compounds may be selected from compounds comprising chloride, fluoride, bromide and iodide ions. In preferred embodiments, suitable cationic halogen containing compounds include, but are not limited to, cetylpyridinium halides, cetyltrimethylammonium halides, cetyldimethylethylammonium halides, cetyldimethylbenzylammonium halides, cetyltributylphosphonium halides, dodecyltrimethylammonium halides, or tetradecyltrimethylammonium halides. In some particular embodiments, suitable cationic halogen containing compounds comprise, but are not limited to, cetylpyridinium chloride (CPC), cetyltrimethylammonium chloride, cetylbenzyltrimethylammonium chloride, cetylpyridinium bromide (CPB), and cetyltrimethylammonium bromide (CTAB), cetyldimethylethylammonium bromide, cetyltributylphosphonium bromide, dodecyltrimethylammonium bromide, and tetradecyltrimethylammonium bromide. In particularly preferred embodiments, the cationic halogen-containing compound is CPC, although the compositions of the present invention are not limited to formulation with any particular cationic containing compound.

5. Germination Enhancers

In other embodiments of the present invention, the nanoemulsions further comprise a germination enhancer. In some preferred embodiments, the emulsions comprise from about 1 mM to 15 mM, and more preferably from about 5 mM to 10 mM of one or more germination enhancing compounds (although other concentrations are also contemplated). In preferred embodiments, the germination enhancing compound is provided in the aqueous phase prior to formation of the emulsion. The present invention contemplates that when germination enhancers are added to the nanoemulsion compositions, the sporicidal properties of the nanoemulsions are enhanced. The present invention further contemplates that such germination enhancers initiate sporicidal activity near neutral pH (between pH 6 - 8, and preferably 7). Such neutral pH emulsions can be obtained, for example, by diluting with phosphate buffer saline (PBS) or by preparations of neutral emulsions. The sporicidal activity of the nanoemulsion preferentially occurs when the spores initiate germination.

In specific embodiments, it has been demonstrated that the emulsions utilized in the vaccines of the present invention have sporicidal activity. While the present invention is not limited to any particular mechanism and an understanding of the mechanism is not required to practice the present invention, it is believed that the fusigenic component of the emulsions acts to initiate germination and before reversion to the vegetative form is complete the lysogenic component of the emulsion acts to lyse the newly germinating spore.

These components of the emulsion thus act in concert to leave the spore susceptible to disruption by the emulsions. The addition of germination enhancer further facilitates the anti-sporicidal activity of the emulsions, for example, by speeding up the rate at which the sporicidal activity occurs.

Germination of bacterial endospores and fungal spores is associated with increased metabolism and decreased resistance to heat and chemical reactants. For germination to occur, the spore must sense that the environment is adequate to support vegetation and reproduction. The amino acid L-alanine stimulates bacterial spore germination (*See e.g.*, Hills, J. Gen. Micro. 4:38 (1950); and Halvorson and Church, Bacteriol Rev. 21:112 (1957)). L-alanine and L-proline have also been reported to initiate fungal spore germination (Yanagita, Arch Mikrobiol 26:329 (1957)). Simple α -amino acids, such as glycine and L-alanine, occupy a central position in metabolism. Transamination or deamination of α -amino acids yields the glycogenic or ketogenic carbohydrates and the nitrogen needed for metabolism and growth. For example, transamination or deamination

of L-alanine yields pyruvate, which is the end product of glycolytic metabolism (Embden-Meyerhof-Parnas Pathway). Oxidation of pyruvate by pyruvate dehydrogenase complex yields acetyl-CoA, NADH, H^+ , and CO_2 . Acetyl-CoA is the initiator substrate for the tricarboxylic acid cycle (Kreb's Cycle), which in turn feeds the mitochondrial electron transport chain. Acetyl-CoA is also the ultimate carbon source for fatty acid synthesis as well as for sterol synthesis. Simple α -amino acids can provide the nitrogen, CO_2 , glycogenic and/or ketogenic equivalents required for germination and the metabolic activity that follows.

In certain embodiments, suitable germination enhancing agents of the invention include, but are not limited to, α -amino acids comprising glycine and the L-enantiomers of alanine, valine, leucine, isoleucine, serine, threonine, lysine, phenylalanine, tyrosine, and the alkyl esters thereof. Additional information on the effects of amino acids on germination may be found in U.S. Pat. No. 5,510,104; herein incorporated by reference in its entirety. In some embodiments, a mixture of glucose, fructose, asparagine, sodium chloride (NaCl), ammonium chloride (NH_4Cl), calcium chloride ($CaCl_2$) and potassium chloride (KCl) also may be used. In particularly preferred embodiments of the present invention, the formulation comprises the germination enhancers L-alanine, $CaCl_2$, Inosine and NH_4Cl . In some embodiments, the compositions further comprise one or more common forms of growth media (*e.g.*, trypticase soy broth, and the like) that additionally may or may not itself comprise germination enhancers and buffers.

The above compounds are merely exemplary germination enhancers and it is understood that other known germination enhancers will find use in the nanoemulsions utilized in some embodiments of the present invention. A candidate germination enhancer should meet two criteria for inclusion in the compositions of the present invention: it should be capable of being associated with the emulsions disclosed herein and it should increase the rate of germination of a target spore when incorporated in the emulsions disclosed herein. One skilled in the art can determine whether a particular agent has the desired function of acting as a germination enhancer by applying such an agent in combination with the nanoemulsions disclosed herein to a target and comparing the inactivation of the target when contacted by the admixture with inactivation of like targets by the composition of the present invention without the agent. Any agent that increases germination, and thereby decreases or inhibits the growth of the organisms, is considered a

suitable enhancer for use in the nanoemulsion compositions disclosed herein.

In still other embodiments, addition of a germination enhancer (or growth medium) to a neutral emulsion composition produces a composition that is useful in inactivating bacterial spores in addition to enveloped viruses, Gram negative bacteria, and Gram positive bacteria for use in the vaccine compositions of the present invention.

6. Interaction Enhancers

In still other embodiments, nanoemulsions comprise one or more compounds capable of increasing the interaction of the compositions (*i.e.*, "interaction enhancer") with target pathogens (*e.g.*, the cell wall of Gram negative bacteria such as *Vibrio*, *Salmonella*, *Shigella* and *Pseudomonas*). In preferred embodiments, the interaction enhancer is preferably premixed with the oil phase; however, in other embodiments the interaction enhancer is provided in combination with the compositions after emulsification. In certain preferred embodiments, the interaction enhancer is a chelating agent (*e.g.*, ethylenediaminetetraacetic acid (EDTA) or ethylenebis(oxyethylenitrilo)tetraacetic acid (EGTA) in a buffer (*e.g.*, tris buffer)). It is understood that chelating agents are merely exemplary interaction enhancing compounds. Indeed, other agents that increase the interaction of the nanoemulsions used in some embodiments of the present invention with microbial agents and/or pathogens are contemplated. In particularly preferred embodiments, the interaction enhancer is at a concentration of about 50 to about 250 μM . One skilled in the art will be able to determine whether a particular agent has the desired function of acting as an interaction enhancer by applying such an agent in combination with the compositions of the present invention to a target and comparing the inactivation of the target when contacted by the admixture with inactivation of like targets by the composition of the present invention without the agent. Any agent that increases the interaction of an emulsion with bacteria and thereby decreases or inhibits the growth of the bacteria, in comparison to that parameter in its absence, is considered an interaction enhancer.

In some embodiments, the addition of an interaction enhancer to nanoemulsion produces a composition that is useful in inactivating enveloped viruses, some Gram positive bacteria and some Gram negative bacteria for use in the vaccine compositions of the present invention.

7. Quaternary Ammonium Compounds

In some embodiments, nanoemulsions of the present invention include a quaternary ammonium containing compound. Exemplary quaternary ammonium compounds include, but are not limited to, Alkyl dimethyl benzyl ammonium chloride, didecyl dimethyl ammonium chloride, Alkyl dimethyl benzyl and dialkyl dimethyl ammonium chloride, N,N-Dimethyl-2-hydroxypropylammonium chloride polymer, Didecyl dimethyl ammonium chloride, n-Alkyl dimethyl benzyl ammonium chloride, n-Alkyl dimethyl ethylbenzyl ammonium chloride,

Dialkyl dimethyl ammonium chloride, n-Alkyl dimethyl benzyl ammonium chloride, n-Tetradecyl dimethyl benzyl ammonium chloride monohydrate, n-Alkyl dimethyl benzyl ammonium chloride, Dialkyl dimethyl ammonium chloride, Hexahydro-1,3,5 – tris(2-hydroxyethyl)-s-triazine, Myristalkonium chloride (and) Quat RNIUM 14, Alkyl bis(2-hydroxyethyl) benzyl ammonium chloride, Alkyl demethyl benzyl ammonium chloride, Alkyl dimethyl 3,4-dichlorobenzyl ammonium chloride, Alkyl dimethyl benzyl ammonium chloride, Alkyl dimethyl benzyl dimethylbenzyl ammonium, Alkyl dimethyl dimethylbenzyl ammonium chloride, Alkyl dimethyl ethyl ammonium bromide, Alkyl dimethyl ethyl ammonium bromide, Alkyl dimethyl ethylbenzyl ammonium chloride, Alkyl dimethyl isopropylbenzyl ammonium chloride, Alkyl trimethyl ammonium chloride, Alkyl 1 or 3 benzyl-1-(2-hydroxyethyl)-2-imidazolinium chloride, Dialkyl methyl benzyl ammonium chloride, Dialkyl dimethyl ammonium chloride, Didecyl dimethyl ammonium chloride, 2-(2-(p-(Diisobutyl)cresosy)ethoxy)ethyl dimethyl benzyl ammonium chloride, 2-(2-(p-(Diisobutyl)phenoxy)ethoxy)ethyl dimethyl benzyl ammonium chloride, Dioctyl dimethyl ammonium chloride, Dodecyl bis (2-hydroxyethyl) octyl hydrogen ammonium chloride, Dodecyl dimethyl benzyl ammonium chloride, Dodecylcarbamoyl methyl dinethyl benzyl ammonium chloride, Heptadecyl hydroxyethylimidazolinium chloride, Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine, Octyl decyl dimethyl ammonium chloride, Octyl dodecyl dimethyl ammonium chloride, Octyphenoxyethoxyethyl dimethyl benzyl ammonium chloride, Oxydiethylenebis(alkyl dimethyl ammonium chloride), Quaternary ammonium compounds, dicoco alkyl dimethyl, chloride, Trimethoxysilyl quats, and Trimethyl dodecylbenzyl ammonium chloride.

8. Other Components

In some embodiments, a nanoemulsion comprises one or more additional components that provide a desired property or functionality to the nanoemulsions. These components may be incorporated into the aqueous phase or the oil phase of the

nanoemulsions and/or may be added prior to or following emulsification. For example, in some embodiments, the nanoemulsions further comprise phenols (e.g., triclosan, phenyl phenol), acidifying agents (e.g., citric acid (e.g., 1.5-6%), acetic acid, lemon juice), alkylating agents (e.g., sodium hydroxide (e.g., 0.3%)), buffers (e.g., citrate buffer, acetate buffer, and other buffers useful to maintain a specific pH), and halogens (e.g., polyvinylpyrrolidone, sodium hypochlorite, hydrogen peroxide).

Exemplary techniques for making a nanoemulsion (e.g., used to inactivate a pathogen and/or generation of an immunogenic composition of the present invention) are described below. Additionally, a number of specific, although exemplary, formulation recipes are also set forth below.

Formulation Techniques

Nanoemulsions of the present invention can be formed using classic emulsion forming techniques. In brief, the oil phase is mixed with the aqueous phase under relatively high shear forces (e.g., using high hydraulic and mechanical forces) to obtain an oil-in-water nanoemulsion. The emulsion is formed by blending the oil phase with an aqueous phase on a volume-to-volume basis ranging from about 1:9 to 5:1, preferably about 5:1 to 3:1, most preferably 4:1, oil phase to aqueous phase. The oil and aqueous phases can be blended using any apparatus capable of producing shear forces sufficient to form an emulsion such as French Presses or high shear mixers (e.g., FDA approved high shear mixers are available, for example, from Admix, Inc., Manchester, NH). Methods of producing such emulsions are described in U.S. Pat. Nos. 5,103,497 and 4,895,452, herein incorporated by reference in their entireties.

In preferred embodiments, compositions used in the methods of the present invention comprise droplets of an oily discontinuous phase dispersed in an aqueous continuous phase, such as water. In preferred embodiments, nanoemulsions of the present invention are stable, and do not decompose even after long storage periods (e.g., greater than one or more years). Furthermore, in some embodiments, nanoemulsions are stable (e.g., in some embodiments for greater than 3 months, in some embodiments for greater than 6 months, in some embodiments for greater than 12 months, in some embodiments for greater than 18 months) after combination with an immunogen (e.g., a pathogen). In preferred embodiments, nanoemulsions of the present invention are non-toxic and safe when administered (e.g., via spraying or contacting mucosal surfaces, swallowed, inhaled, etc.) to a subject.

In some embodiments, a portion of the emulsion may be in the form of lipid structures including, but not limited to, unilamellar, multilamellar, and paucilamellar lipid vesicles, micelles, and lamellar phases.

Some embodiments of the present invention employ an oil phase containing ethanol.

For example, in some embodiments, the emulsions of the present invention contain (i) an aqueous phase and (ii) an oil phase containing ethanol as the organic solvent and optionally a germination enhancer, and (iii) TYLOXAPOL as the surfactant (preferably 2-5%, more preferably 3%). This formulation is highly efficacious for inactivation of pathogens and is also non-irritating and non-toxic to mammalian subjects (e.g., and thus can be used for administration to a mucosal surface).

In some other embodiments, the emulsions of the present invention comprise a first emulsion emulsified within a second emulsion, wherein (a) the first emulsion comprises (i) an aqueous phase; and (ii) an oil phase comprising an oil and an organic solvent; and (iii) a surfactant; and (b) the second emulsion comprises (i) an aqueous phase; and (ii) an oil phase comprising an oil and a cationic containing compound; and (iii) a surfactant.

Exemplary Formulations

The following description provides a number of exemplary emulsions including formulations for compositions BCTP and $X_8W_{60}PC$. BCTP comprises a water-in oil nanoemulsion, in which the oil phase was made from soybean oil, tri-n-butyl phosphate, and TRITON X-100 in 80% water. $X_8W_{60}PC$ comprises a mixture of equal volumes of BCTP with $W_{80}8P$. $W_{80}8P$ is a liposome-like compound made of glycerol monostearate, refined soya sterols (e.g., GENEROL sterols), TWEEN 60, soybean oil, a cationic ion halogen-containing CPC and peppermint oil. The GENEROL family are a group of a polyethoxylated soya sterols (Henkel Corporation, Ambler, Pennsylvania). Exemplary emulsion formulations useful in the present invention are provided in Table 1B. These particular formulations may be found in U.S. Pat. Nos. 5,700,679 (NN); 5,618,840; 5,549,901 ($W_{80}8P$); and 5,547,677, each of which is hereby incorporated by reference in their entireties. Certain other emulsion formulations are presented U.S. Pat. App. Serial No. 10/669,865, hereby incorporated by reference in its entirety.

The $X_8W_{60}PC$ emulsion is manufactured by first making the $W_{80}8P$ emulsion and BCTP emulsions separately. A mixture of these two emulsions is then re-emulsified to produce a fresh emulsion composition termed $X_8W_{60}PC$. Methods of producing such

emulsions are described in U.S. Pat. Nos. 5,103,497 and 4,895,452 (each of which is herein incorporated by reference in their entireties).

Table 1B

	Oil Phase Formula	Water to Oil Phase Ratio (Vol/Vol)
BCTP	1 vol. Tri(N-butyl)phosphate 1 vol. TRITON X-100 8 vol. Soybean oil	4:1
NN	86.5 g Glycerol monooleate 60.1 ml Nonoxynol-9 24.2 g GENEROL 122 3.27 g Cetylpyridinium chloride 554 g Soybean oil	3:1
W ₈₀ 8P	86.5 g Glycerol monooleate 21.2 g Polysorbate 60 24.2 g GENEROL 122 3.27 g Cetylpyddinium chloride 4 ml Peppermint oil 554 g Soybean oil	3.2:1
SS	86.5 g Glycerol monooleate 21.2 g Polysorbate 60 24.2 g GENEROL 122 3.27 g Cetylpyridinium chloride 554 g Soybean oil	3.2:1 (1% bismuth in water)

The compositions listed above are only exemplary and those of skill in the art will be able to alter the amounts of the components to arrive at a nanoemulsion composition suitable for the purposes of the present invention. Those skilled in the art will understand that the ratio of oil phase to water as well as the individual oil carrier, surfactant CPC and organic phosphate buffer, components of each composition may vary.

Although certain compositions comprising BCTP have a water to oil ratio of 4:1, it is understood that the BCTP may be formulated to have more or less of a water phase. For

example, in some embodiments, there is 3, 4, 5, 6, 7, 8, 9, 10, or more parts of the water phase to each part of the oil phase. The same holds true for the W₈₀8P formulation. Similarly, the ratio of Tri(N-butyl)phosphate:TRITON X-100:soybean oil also may be varied.

Although Table 1B lists specific amounts of glycerol monooleate, polysorbate 60, GENEROL 122, cetylpyridinium chloride, and carrier oil for W₈₀8P, these are merely exemplary. An emulsion that has the properties of W₈₀8P may be formulated that has different concentrations of each of these components or indeed different components that will fulfill the same function. For example, the emulsion may have between about 80 to about 100g of glycerol monooleate in the initial oil phase. In other embodiments, the emulsion may have between about 15 to about 30 g polysorbate 60 in the initial oil phase. In yet another embodiment the composition may comprise between about 20 to about 30 g of a GENEROL sterol, in the initial oil phase.

Individual components of nanoemulsions (e.g. in an immunogenic composition of the present invention) can function both to inactivate a pathogen as well as to contribute to the non-toxicity of the emulsions. For example, the active component in BCTP, TRITON-X100, shows less ability to inactivate a virus at concentrations equivalent to 11% BCTP. Adding the oil phase to the detergent and solvent markedly reduces the toxicity of these agents in tissue culture at the same concentrations. While not being bound to any theory (an understanding of the mechanism is not necessary to practice the present invention, and the present invention is not limited to any particular mechanism), it is suggested that the nanoemulsion enhances the interaction of its components with the pathogens thereby facilitating the inactivation of the pathogen and reducing the toxicity of the individual components. Furthermore, when all the components of BCTP are combined in one composition but are not in a nanoemulsion structure, the mixture is not as effective at inactivating a pathogen as when the components are in a nanoemulsion structure.

Numerous additional embodiments presented in classes of formulations with like compositions are presented below. The following compositions recite various ratios and mixtures of active components. One skilled in the art will appreciate that the below recited formulation are exemplary and that additional formulations comprising similar percent ranges of the recited components are within the scope of the present invention.

In certain embodiments of the present invention, a nanoemulsion comprises from about 3 to 8 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of

cetylpyridinium chloride (CPC), about 60 to 70 vol. % oil (e.g., soybean oil), about 15 to 25 vol. % of aqueous phase (e.g., DiH_2O or PBS), and in some formulations less than about 1 vol. % of 1N NaOH. Some of these embodiments comprise PBS. It is contemplated that the addition of 1N NaOH and/or PBS in some of these embodiments, allows the user to advantageously control the pH of the formulations, such that pH ranges from about 7.0 to about 9.0, and more preferably from about 7.1 to 8.5 are achieved. For example, one embodiment of the present invention comprises about 3 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 24 vol. % of DiH_2O (designated herein as Y3EC). Another similar embodiment comprises about 3.5 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, and about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 23.5 vol. % of DiH_2O (designated herein as Y3.5EC). Yet another embodiment comprises about 3 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 0.067 vol. % of 1N NaOH, such that the pH of the formulation is about 7.1, about 64 vol. % of soybean oil, and about 23.93 vol. % of DiH_2O (designated herein as Y3EC pH 7.1). Still another embodiment comprises about 3 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 0.67 vol. % of 1N NaOH, such that the pH of the formulation is about 8.5, and about 64 vol. % of soybean oil, and about 23.33 vol. % of DiH_2O (designated herein as Y3EC pH 8.5). Another similar embodiment comprises about 4% TYLOXAPOL, about 8 vol. % ethanol, about 1% CPC, and about 64 vol. % of soybean oil, and about 23 vol. % of DiH_2O (designated herein as Y4EC). In still another embodiment the formulation comprises about 8% TYLOXAPOL, about 8% ethanol, about 1 vol. % of CPC, and about 64 vol. % of soybean oil, and about 19 vol. % of DiH_2O (designated herein as Y8EC). A further embodiment comprises about 8 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 19 vol. % of 1x PBS (designated herein as Y8EC PBS).

In some embodiments of the present invention, a nanoemulsion comprises about 8 vol. % of ethanol, and about 1 vol. % of CPC, and about 64 vol. % of oil (e.g., soybean oil), and about 27 vol. % of aqueous phase (e.g., DiH_2O or PBS) (designated herein as EC).

In some embodiments, a nanoemulsion comprises from about 8 vol. % of sodium dodecyl sulfate (SDS), about 8 vol. % of tributyl phosphate (TBP), and about 64 vol. % of oil (e.g., soybean oil), and about 20 vol. % of aqueous phase (e.g., DiH_2O or PBS) (designated herein as S8P).

In some embodiments, a nanoemulsion comprises from about 1 to 2 vol. % of

TRITON X-100, from about 1 to 2 vol. % of TYLOXAPOL, from about 7 to 8 vol. % of ethanol, about 1 vol. % of cetylpyridinium chloride (CPC), about 64 to 57.6 vol. % of oil (e.g., soybean oil), and about 23 vol. % of aqueous phase (e.g., DiH₂O or PBS).

Additionally, some of these formulations further comprise about 5 mM of L-alanine/Inosine, and about 10 mM ammonium chloride. Some of these formulations comprise PBS. It is contemplated that the addition of PBS in some of these embodiments, allows the user to advantageously control the pH of the formulations. For example, one embodiment of the present invention comprises about 2 vol. % of TRITON X-100, about 2 vol. % of TYLOXAPOL, about 8 vol. % of ethanol, about 1 vol. % CPC, about 64 vol. % of soybean oil, and about 23 vol. % of aqueous phase DiH₂O. In another embodiment the formulation comprises about 1.8 vol. % of TRITON X-100, about 1.8 vol. % of TYLOXAPOL, about 7.2 vol. % of ethanol, about 0.9 vol. % of CPC, about 5 mM L-alanine/Inosine, and about 10 mM ammonium chloride, about 57.6 vol. % of soybean oil, and the remainder of 1x PBS (designated herein as 90% X2Y2EC/GE).

In alternative embodiments, a nanoemulsion comprises from about 5 vol. % of TWEEN 80, from about 8 vol. % of ethanol, from about 1 vol. % of CPC, about 64 vol. % of oil (e.g., soybean oil), and about 22 vol. % of DiH₂O (designated herein as W₈₀5EC).

In still other embodiments of the present invention, a nanoemulsion comprises from about 5 vol. % of TWEEN 20, from about 8 vol. % of ethanol, from about 1 vol. % of CPC, about 64 vol. % of oil (e.g., soybean oil), and about 22 vol. % of DiH₂O (designated herein as W₂₀5EC).

In still other embodiments of the present invention, a nanoemulsion comprises from about 2 to 8 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 1 vol. % of CPC, about 60 to 70 vol. % of oil (e.g., soybean, or olive oil), and about 15 to 25 vol. % of aqueous phase (e.g., DiH₂O or PBS). For example, the present invention contemplates formulations comprising about 2 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 26 vol. % of DiH₂O (designated herein as X2E). In other similar embodiments, a nanoemulsion comprises about 3 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 25 vol. % of DiH₂O (designated herein as X3E). In still further embodiments, the formulations comprise about 4 vol. % Triton of X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 24 vol. % of DiH₂O (designated herein as X4E). In yet other embodiments, a nanoemulsion comprises about 5 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of

soybean oil, and about 23 vol. % of DiH₂O (designated herein as X5E). In some embodiments, a nanoemulsion comprises about 6 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 22 vol. % of DiH₂O (designated herein as X6E). In still further embodiments of the present invention, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as X8E). In still further embodiments, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of ethanol, about 64 vol. % of olive oil, and about 20 vol. % of DiH₂O (designated herein as X8E O). In yet another embodiment, a nanoemulsion comprises 8 vol. % of TRITON X-100, about 8 vol. % ethanol, about 1 vol. % CPC, about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O (designated herein as X8EC).

In alternative embodiments of the present invention, a nanoemulsion comprises from about 1 to 2 vol. % of TRITON X-100, from about 1 to 2 vol. % of TYLOXAPOL, from about 6 to 8 vol. % TBP, from about 0.5 to 1.0 vol. % of CPC, from about 60 to 70 vol. % of oil (e.g., soybean), and about 1 to 35 vol. % of aqueous phase (e.g., DiH₂O or PBS).

Additionally, certain of these nanoemulsions may comprise from about 1 to 5 vol. % of trypticase soy broth, from about 0.5 to 1.5 vol. % of yeast extract, about 5 mM L-alanine/Inosine, about 10 mM ammonium chloride, and from about 20-40 vol. % of liquid baby formula. In some embodiments comprising liquid baby formula, the formula

comprises a casein hydrolysate (e.g., Neutramigen, or Progestimil, and the like). In some of these embodiments, a nanoemulsion further comprises from about 0.1 to 1.0 vol. % of sodium thiosulfate, and from about 0.1 to 1.0 vol. % of sodium citrate. Other similar embodiments comprising these basic components employ phosphate buffered saline (PBS) as the aqueous phase. For example, one embodiment comprises about 2 vol. % of TRITON X-100, about 2 vol. % TYLOXAPOL, about 8 vol. % TBP, about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 23 vol. % of DiH₂O (designated herein as X2Y2EC). In still other embodiments, the inventive formulation comprises about 2 vol. % of TRITON X-100, about 2 vol. % TYLOXAPOL, about 8 vol. % TBP, about 1 vol. % of CPC, about 0.9 vol. % of sodium thiosulfate, about 0.1 vol. % of sodium citrate, about 64 vol. % of soybean oil, and about 22 vol. % of DiH₂O (designated herein as X2Y2PC STS1). In another similar embodiment, a nanoemulsion comprises about 1.7 vol. % TRITON X-100, about 1.7 vol. % TYLOXAPOL, about 6.8 vol. % TBP, about 0.85% CPC, about 29.2% NEUTRAMIGEN, about 54.4 vol. % of soybean oil, and about 4.9 vol. % of DiH₂O (designated herein as 85%

X2Y2PC/baby). In yet another embodiment of the present invention, a nanoemulsion comprises about 1.8 vol. % of TRITON X-100, about 1.8 vol. % of TYLOXAPOL, about 7.2 vol. % of TBP, about 0.9 vol. % of CPC, about 5mM L-alanine/Inosine, about 10mM ammonium chloride, about 57.6 vol. % of soybean oil, and the remainder vol. % of 0.1x PBS (designated herein as 90% X2Y2 PC/GE). In still another embodiment, a nanoemulsion comprises about 1.8 vol. % of TRITON X-100, about 1.8 vol. % of TYLOXAPOL, about 7.2 vol. % TBP, about 0.9 vol. % of CPC, and about 3 vol. % trypticase soy broth, about 57.6 vol. % of soybean oil, and about 27.7 vol. % of DiH₂O (designated herein as 90% X2Y2PC/TSB). In another embodiment of the present invention, a nanoemulsion comprises about 1.8 vol. % TRITON X-100, about 1.8 vol. % TYLOXAPOL, about 7.2 vol. % TBP, about 0.9 vol. % CPC, about 1 vol. % yeast extract, about 57.6 vol. % of soybean oil, and about 29.7 vol. % of DiH₂O (designated herein as 90% X2Y2PC/YE).

In some embodiments of the present invention, a nanoemulsion comprises about 3 vol. % of TYLOXAPOL, about 8 vol. % of TBP, and about 1 vol. % of CPC, about 60 to 70 vol. % of oil (e.g., soybean or olive oil); and about 15 to 30 vol. % of aqueous phase (e.g., DiH₂O or PBS). In a particular embodiment of the present invention, a nanoemulsion comprises about 3 vol. % of TYLOXAPOL, about 8 vol. % of TBP, and about 1 vol. % of CPC, about 64 vol. % of soybean, and about 24 vol. % of DiH₂O (designated herein as Y3PC).

In some embodiments of the present invention, a nanoemulsion comprises from about 4 to 8 vol. % of TRITON X-100, from about 5 to 8 vol. % of TBP, about 30 to 70 vol. % of oil (e.g., soybean or olive oil), and about 0 to 30 vol. % of aqueous phase (e.g., DiH₂O or PBS). Additionally, certain of these embodiments further comprise about 1 vol. % of CPC, about 1 vol. % of benzalkonium chloride, about 1 vol. % cetylridinium bromide, about 1 vol. % cetyldimethyletylammonium bromide, 500 μ M EDTA, about 10 mM ammonium chloride, about 5 mM Inosine, and about 5 mM L-alanine. For example, in a certain preferred embodiment, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as X8P). In another embodiment of the present invention, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1% of CPC, about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O (designated herein as X8PC). In still another embodiment, a nanoemulsion comprises about 8 vol. %

TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of CPC, about 50 vol. % of soybean oil, and about 33 vol. % of DiH₂O (designated herein as ATB-X1001). In yet another embodiment, the formulations comprise about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 2 vol. % of CPC, about 50 vol. % of soybean oil, and about 32 vol. % of DiH₂O (designated herein as ATB-X002). In some embodiments, a nanoemulsion comprises about 4 vol. % TRITON X-100, about 4 vol. % of TBP, about 0.5 vol. % of CPC, about 32 vol. % of soybean oil, and about 59.5 vol. % of DiH₂O (designated herein as 50% X8PC). In some embodiments, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 0.5 vol. % CPC, about 64 vol. % of soybean oil, and about 19.5 vol. % of DiH₂O (designated herein as X8PC_{1/2}). In some embodiments of the present invention, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 2 vol. % of CPC, about 64 vol. % of soybean oil, and about 18 vol. % of DiH₂O (designated herein as X8PC2). In other embodiments, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8% of TBP, about 1% of benzalkonium chloride, about 50 vol. % of soybean oil, and about 33 vol. % of DiH₂O (designated herein as X8P BC). In an alternative embodiment of the present invention, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of cetylridinium bromide, about 50 vol. % of soybean oil, and about 33 vol. % of DiH₂O (designated herein as X8P CPB). In another exemplary embodiment of the present invention, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of cetyldimethyletylammonium bromide, about 50 vol. % of soybean oil, and about 33 vol. % of DiH₂O (designated herein as X8P CTAB). In still further embodiments, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of CPC, about 500 μ M EDTA, about 64 vol. % of soybean oil, and about 15.8 vol. % DiH₂O (designated herein as X8PC EDTA). In some embodiments, a nanoemulsion comprises 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 1 vol. % of CPC, about 10 mM ammonium chloride, about 5mM Inosine, about 5mM L-alanine, about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O or PBS (designated herein as X8PC GE_{1x}). In another embodiment of the present invention, a nanoemulsion comprises about 5 vol. % of TRITON X-100, about 5% of TBP, about 1 vol. % of CPC, about 40 vol. % of soybean oil, and about 49 vol. % of DiH₂O (designated herein as X5P₅C).

In some embodiments of the present invention, a nanoemulsion comprises about 2 vol. % TRITON X-100, about 6 vol. % TYLOXAPOL, about 8 vol. % ethanol, about 64

vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as X2Y6E).

In an additional embodiment of the present invention, a nanoemulsion comprises about 8 vol. % of TRITON X-100, and about 8 vol. % of glycerol, about 60 to 70 vol. % of oil (e.g., soybean or olive oil), and about 15 to 25 vol. % of aqueous phase (e.g., DiH₂O or PBS). Certain nanoemulsion compositions (e.g., used to generate an immune response (e.g., for use as a vaccine) comprise about 1 vol. % L-ascorbic acid. For example, one particular embodiment comprises about 8 vol. % of TRITON X-100, about 8 vol. % of glycerol, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as X8G). In still another embodiment, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 8 vol. % of glycerol, about 1 vol. % of L-ascorbic acid, about 64 vol. % of soybean oil, and about 19 vol. % of DiH₂O (designated herein as X8GV_c).

In still further embodiments, a nanoemulsion comprises about 8 vol. % of TRITON X-100, from about 0.5 to 0.8 vol. % of TWEEN 60, from about 0.5 to 2.0 vol. % of CPC, about 8 vol. % of TBP, about 60 to 70 vol. % of oil (e.g., soybean or olive oil), and about 15 to 25 vol. % of aqueous phase (e.g., DiH₂O or PBS). For example, in one particular embodiment a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 0.70 vol. % of TWEEN 60, about 1 vol. % of CPC, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 18.3 vol. % of DiH₂O (designated herein as X8W60PC₁). In some embodiments, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 0.71 vol. % of TWEEN 60, about 1 vol. % of CPC, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 18.29 vol. % of DiH₂O (designated herein as W60_{0.7}X8PC). In yet other embodiments, a nanoemulsion comprises from about 8 vol. % of TRITON X-100, about 0.7 vol. % of TWEEN 60, about 0.5 vol. % of CPC, about 8 vol. % of TBP, about 64 to 70 vol. % of soybean oil, and about 18.8 vol. % of DiH₂O (designated herein as X8W60PC₂). In still other embodiments, a nanoemulsion comprises about 8 vol. % of TRITON X-100, about 0.71 vol. % of TWEEN 60, about 2 vol. % of CPC, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 17.3 vol. % of DiH₂O. In another embodiment of the present invention, a nanoemulsion comprises about 0.71 vol. % of TWEEN 60, about 1 vol. % of CPC, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 25.29 vol. % of DiH₂O (designated herein as W60_{0.7}PC).

In another embodiment of the present invention, a nanoemulsion comprises about 2 vol. % of dioctyl sulfosuccinate, either about 8 vol. % of glycerol, or about 8 vol. % TBP, in addition to, about 60 to 70 vol. % of oil (e.g., soybean or olive oil), and about 20 to 30 vol.

% of aqueous phase (e.g., DiH_2O or PBS). For example, in some embodiments, a nanoemulsion comprises about 2 vol. % of dioctyl sulfosuccinate, about 8 vol. % of glycerol, about 64 vol. % of soybean oil, and about 26 vol. % of DiH_2O (designated herein as D2G). In another related embodiment, a nanoemulsion comprises about 2 vol. % of dioctyl sulfosuccinate, and about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 26 vol. % of DiH_2O (designated herein as D2P).

In still other embodiments of the present invention, a nanoemulsion comprises about 8 to 10 vol. % of glycerol, and about 1 to 10 vol. % of CPC, about 50 to 70 vol. % of oil (e.g., soybean or olive oil), and about 15 to 30 vol. % of aqueous phase (e.g., DiH_2O or PBS). Additionally, in certain of these embodiments, a nanoemulsion further comprises about 1 vol. % of L-ascorbic acid. For example, in some embodiments, a nanoemulsion comprises about 8 vol. % of glycerol, about 1 vol. % of CPC, about 64 vol. % of soybean oil, and about 27 vol. % of DiH_2O (designated herein as GC). In some embodiments, a nanoemulsion comprises about 10 vol. % of glycerol, about 10 vol. % of CPC, about 60 vol. % of soybean oil, and about 20 vol. % of DiH_2O (designated herein as GC10). In still another embodiment of the present invention, a nanoemulsion comprises about 10 vol. % of glycerol, about 1 vol. % of CPC, about 1 vol. % of L-ascorbic acid, about 64 vol. % of soybean or oil, and about 24 vol. % of DiH_2O (designated herein as GCV_c).

In some embodiments of the present invention, a nanoemulsion comprises about 8 to 10 vol. % of glycerol, about 8 to 10 vol. % of SDS, about 50 to 70 vol. % of oil (e.g., soybean or olive oil), and about 15 to 30 vol. % of aqueous phase (e.g., DiH_2O or PBS). Additionally, in certain of these embodiments, a nanoemulsion further comprise about 1 vol. % of lecithin, and about 1 vol. % of p-Hydroxybenzoic acid methyl ester. Exemplary embodiments of such formulations comprise about 8 vol. % SDS, 8 vol. % of glycerol, about 64 vol. % of soybean oil, and about 20 vol. % of DiH_2O (designated herein as S8G). A related formulation comprises about 8 vol. % of glycerol, about 8 vol. % of SDS, about 1 vol. % of lecithin, about 1 vol. % of p-Hydroxybenzoic acid methyl ester, about 64 vol. % of soybean oil, and about 18 vol. % of DiH_2O (designated herein as S8GL1B1).

In yet another embodiment of the present invention, a nanoemulsion comprises about 4 vol. % of TWEEN 80, about 4 vol. % of TYLOXAPOL, about 1 vol. % of CPC, about 8 vol. % of ethanol, about 64 vol. % of soybean oil, and about 19 vol. % of DiH_2O (designated herein as W₈₀4Y4EC).

In some embodiments of the present invention, a nanoemulsion comprises about

0.01 vol. % of CPC, about 0.08 vol. % of TYLOXAPOL, about 10 vol. % of ethanol, about 70 vol. % of soybean oil, and about 19.91 vol. % of DiH₂O (designated herein as Y.08EC.01).

In yet another embodiment of the present invention, a nanoemulsion comprises about 8 vol. % of sodium lauryl sulfate, and about 8 vol. % of glycerol, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as SLS8G).

The specific formulations described above are simply examples to illustrate the variety of nanoemulsions that find use (e.g., to inactivate and/or neutralize a pathogen, and for generating an immune response in a subject (e.g., for use as a vaccine)) in the present invention. The present invention contemplates that many variations of the above formulations, as well as additional nanoemulsions, find use in the methods of the present invention. Candidate emulsions can be easily tested to determine if they are suitable. First, the desired ingredients are prepared using the methods described herein, to determine if an emulsion can be formed. If an emulsion cannot be formed, the candidate is rejected. For example, a candidate composition made of 4.5% sodium thiosulfate, 0.5% sodium citrate, 10% n-butanol, 64% soybean oil, and 21% DiH₂O does not form an emulsion.

Second, the candidate emulsion should form a stable emulsion. An emulsion is stable if it remains in emulsion form for a sufficient period to allow its intended use (e.g., to generate an immune response in a subject). For example, for emulsions that are to be stored, shipped, etc., it may be desired that the composition remain in emulsion form for months to years. Typical emulsions that are relatively unstable, will lose their form within a day. For example, a candidate composition made of 8% 1-butanol, 5% Tween 10, 1% CPC, 64% soybean oil, and 22% DiH₂O does not form a stable emulsion. Nanoemulsions that have been shown to be stable include, but are not limited to, 8 vol. % of TRITON X-100, about 8 vol. % of TBP, about 64 vol. % of soybean oil, and about 20 vol. % of DiH₂O (designated herein as X8P); 5 vol. % of TWEEN 20, from about 8 vol. % of ethanol, from about 1 vol. % of CPC, about 64 vol. % of oil (e.g., soybean oil), and about 22 vol. % of DiH₂O (designated herein as W₂₀5EC); 0.08% Triton X-100, 0.08% Glycerol, 0.01% Cetylpyridinium Chloride, 99% Butter, and 0.83% diH₂O (designated herein as 1% X8GC Butter); 0.8% Triton X-100, 0.8% Glycerol, 0.1% Cetylpyridinium Chloride, 6.4% Soybean Oil, 1.9% diH₂O, and 90% Butter (designated herein as 10% X8GC Butter); 2% W₂₀5EC, 1% Natrosol 250L NF, and 97% diH₂O (designated herein as 2% W₂₀5EC L GEL); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Ethanol, 64% 70 Viscosity Mineral Oil, and

22% diH₂O (designated herein as W₂₀5EC 70 Mineral Oil); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Ethanol, 64% 350 Viscosity Mineral Oil, and 22% diH₂O (designated herein as W₂₀5EC 350 Mineral Oil). In some embodiments, nanoemulsions of the present invention are stable for over a week, over a month, or over a year.

Third, the candidate emulsion should have efficacy for its intended use. For example, a nanoemulsion should inactivate (e.g., kill or inhibit growth of) a pathogen to a desired level (e.g., 1 log, 2 log, 3 log, 4 log, . . . reduction). Using the methods described herein, one is capable of determining the suitability of a particular candidate emulsion against the desired pathogen. Generally, this involves exposing the pathogen to the emulsion for one or more time periods in a side-by-side experiment with the appropriate control samples (e.g., a negative control such as water) and determining if, and to what degree, the emulsion inactivates (e.g., kills and/or neutralizes) the microorganism. For example, a candidate composition made of 1% ammonium chloride, 5% Tween 20, 8% ethanol, 64% soybean oil, and 22% diH₂O was shown not to be an effective emulsion. The following candidate emulsions were shown to be effective using the methods described herein: 5% Tween 20, 5% Cetylpyridinium Chloride, 10% Glycerol, 60% Soybean Oil, and 20% diH₂O (designated herein as W₂₀5GC5); 1% Cetylpyridinium Chloride, 5% Tween 20, 10% Glycerol, 64% Soybean Oil, and 20% diH₂O (designated herein as W₂₀5GC); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Ethanol, 64% Olive Oil, and 22% diH₂O (designated herein as W₂₀5EC Olive Oil); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Ethanol, 64% Flaxseed Oil, and 22% diH₂O (designated herein as W₂₀5EC Flaxseed Oil); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Ethanol, 64% Corn Oil, and 22% diH₂O (designated herein as W₂₀5EC Corn Oil); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Ethanol, 64% Coconut Oil, and 22% diH₂O (designated herein as W₂₀5EC Coconut Oil); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Ethanol, 64% Cottonseed Oil, and 22% diH₂O (designated herein as W₂₀5EC Cottonseed Oil); 8% Dextrose, 5% Tween 10, 1% Cetylpyridinium Chloride, 64% Soybean Oil, and 22% diH₂O (designated herein as W₂₀5C Dextrose); 8% PEG 200, 5% Tween 10, 1% Cetylpyridinium Chloride, 64% Soybean Oil, and 22% diH₂O (designated herein as W₂₀5C PEG 200); 8% Methanol, 5% Tween 10, 1% Cetylpyridinium Chloride, 64% Soybean Oil, and 22% diH₂O (designated herein as W₂₀5C Methanol); 8% PEG 1000, 5% Tween 10, 1% Cetylpyridinium Chloride, 64% Soybean Oil, and 22% diH₂O (designated herein as W₂₀5C PEG 1000); 2% W₂₀5EC, 2% Natrosol 250H NF, and 96% diH₂O (designated herein as 2% W₂₀5EC Natrosol 2, also called 2% W₂₀5EC

GEL); 2% W₂₀5EC, 1% Natrosol 250H NF, and 97% diH₂O (designated herein as 2% W₂₀5EC Natrosol 1); 2% W₂₀5EC, 3% Natrosol 250H NF, and 95% diH₂O (designated herein as 2% W₂₀5EC Natrosol 3); 2% W₂₀5EC, 0.5% Natrosol 250H NF, and 97.5% diH₂O (designated herein as 2% W₂₀5EC Natrosol 0.5); 2% W₂₀5EC, 2% Methocel A, and 96% diH₂O (designated herein as 2% W₂₀5EC Methocel A); 2% W₂₀5EC, 2% Methocel K, and 96% diH₂O (designated herein as 2% W₂₀5EC Methocel K); 2% Natrosol, 0.1% X8PC, 0.1x PBS, 5 mM L-alanine, 5 mM Inosine, 10 mM Ammonium Chloride, and diH₂O (designated herein as 0.1% X8PC/GE+2% Natrosol); 2% Natrosol, 0.8% Triton X-100, 0.8% Tributyl Phosphate, 6.4% Soybean Oil, 0.1% Cetylpyridinium Chloride, 0.1x PBS, 5 mM L-alanine, 5 mM Inosine, 10 mM Ammonium Chloride, and diH₂O (designated herein as 10% X8PC/GE+2% Natrosol); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Ethanol, 64% Lard, and 22% diH₂O (designated herein as W₂₀5EC Lard); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Ethanol, 64% Mineral Oil, and 22% diH₂O (designated herein as W₂₀5EC Mineral Oil); 0.1% Cetylpyridinium Chloride, 2% Nerolidol, 5% Tween 20, 10% Ethanol, 64% Soybean Oil, and 18.9% diH₂O (designated herein as W₂₀5EC_{0.1}N); 0.1% Cetylpyridinium Chloride, 2% Farnesol, 5% Tween 20, 10% Ethanol, 64% Soybean Oil, and 18.9% diH₂O (designated herein as W₂₀5EC_{0.1}F); 0.1% Cetylpyridinium Chloride, 5% Tween 20, 10% Ethanol, 64% Soybean Oil, and 20.9% diH₂O (designated herein as W₂₀5EC_{0.1}); 10% Cetylpyridinium Chloride, 8% Tributyl Phosphate, 8% Triton X-100, 54% Soybean Oil, and 20% diH₂O (designated herein as X8PC₁₀); 5% Cetylpyridinium Chloride, 8% Triton X-100, 8% Tributyl Phosphate, 59% Soybean Oil, and 20% diH₂O (designated herein as X8PC₅); 0.02% Cetylpyridinium Chloride, 0.1% Tween 20, 10% Ethanol, 70% Soybean Oil, and 19.88% diH₂O (designated herein as W₂₀0.1EC_{0.02}); 1% Cetylpyridinium Chloride, 5% Tween 20, 8% Glycerol, 64% Mobil 1, and 22% diH₂O (designated herein as W₂₀5GC Mobil 1); 7.2% Triton X-100, 7.2% Tributyl Phosphate, 0.9% Cetylpyridinium Chloride, 57.6% Soybean Oil, 0.1x PBS, 5 mM L-alanine, 5 mM Inosine, 10 mM Ammonium Chloride, and 25.87% diH₂O (designated herein as 90% X8PC/GE); 7.2% Triton X-100, 7.2% Tributyl Phosphate, 0.9% Cetylpyridinium Chloride, 57.6% Soybean Oil, 1% EDTA, 5 mM L-alanine, 5 mM Inosine, 10 mM Ammonium Chloride, 0.1x PBS, and diH₂O (designated herein as 90% X8PC/GE EDTA); and 7.2% Triton X-100, 7.2% Tributyl Phosphate, 0.9% Cetylpyridinium Chloride, 57.6% Soybean Oil, 1% Sodium Thiosulfate, 5 mM L-alanine, 5 mM Inosine, 10 mM Ammonium Chloride, 0.1x PBS, and diH₂O (designated herein as 90% X8PC/GE STS).

In preferred embodiments of the present invention, the nanoemulsions are non-toxic (e.g., to humans, plants, or animals), non-irritant (e.g., to humans, plants, or animals), and non-corrosive (e.g., to humans, plants, or animals or the environment), while possessing potency against a broad range of microorganisms including bacteria, fungi, viruses, and spores. While a number of the above described nanoemulsions meet these qualifications, the following description provides a number of preferred non-toxic, non-irritant, non-corrosive, anti-microbial nanoemulsions of the present invention (hereinafter in this section referred to as "non-toxic nanoemulsions").

In some embodiments the non-toxic nanoemulsions comprise surfactant lipid preparations (SLPs) for use as broad-spectrum antimicrobial agents that are effective against bacteria and their spores, enveloped viruses, and fungi. In preferred embodiments, these SLPs comprises a mixture of oils, detergents, solvents, and cationic halogen-containing compounds in addition to several ions that enhance their biocidal activities. These SLPs are characterized as stable, non-irritant, and non-toxic compounds compared to commercially available bactericidal and sporicidal agents, which are highly irritant and/or toxic.

Ingredients for use in the non-toxic nanoemulsions include, but are not limited to: detergents (e.g., TRITON X-100 (5-15%) or other members of the TRITON family, TWEEN 60 (0.5-2%) or other members of the TWEEN family, or TYLOXAPOL (1-10%)); solvents (e.g., tributyl phosphate (5-15%)); alcohols (e.g., ethanol (5-15%) or glycerol (5-15%)); oils (e.g., soybean oil (40-70%)); cationic halogen-containing compounds (e.g., cetylpyridinium chloride (0.5-2%), cetylpyridinium bromide (0.5-2%)), or cetyldimethylethyl ammonium bromide (0.5-2%)); quaternary ammonium compounds (e.g., benzalkonium chloride (0.5-2%), N-alkyldimethylbenzyl ammonium chloride (0.5-2%)); ions (calcium chloride (1mM-40mM), ammonium chloride (1mM-20mM), sodium chloride (5mM-200mM), sodium phosphate (1mM-20mM)); nucleosides (e.g., inosine (50μM-20mM)); and amino acids (e.g., L-alanine (50μM-20mM)). Emulsions are prepared, for example, by mixing in a high shear mixer for 3-10 minutes. The emulsions may or may not be heated before mixing at 82°C for 1 hour.

Quaternary ammonium compounds for use in the present include, but are not limited to, N-alkyldimethyl benzyl ammonium saccharinate; 1,3,5-Triazine-1,3,5(2H,4H,6H)-triethanol; 1-Decanaminium, N-decyl-N, N-dimethyl-, chloride (or) Didecyl dimethyl ammonium chloride; 2-(2-(p-(Diisobutyl)cresosy)ethoxy)ethyl dimethyl benzyl ammonium chloride; 2-(2-(p-(Diisobutyl)phenoxy)ethoxy)ethyl dimethyl benzyl ammonium chloride;

alkyl 1 or 3 benzyl-1-(2-hydroxyethyl)-2-imidazolinium chloride; alkyl bis(2-hydroxyethyl) benzyl ammonium chloride; alkyl demethyl benzyl ammonium chloride; alkyl dimethyl 3,4-dichlorobenzyl ammonium chloride (100% C12); alkyl dimethyl 3,4-dichlorobenzyl ammonium chloride (50% C14, 40% C12, 10% C16); alkyl dimethyl 3,4-dichlorobenzyl ammonium chloride (55% C14, 23% C12, 20% C16); alkyl dimethyl benzyl ammonium chloride; alkyl dimethyl benzyl ammonium chloride (100% C14); alkyl dimethyl benzyl ammonium chloride (100% C16); alkyl dimethyl benzyl ammonium chloride (41% C14, 28% C12); alkyl dimethyl benzyl ammonium chloride (47% C12, 18% C14); alkyl dimethyl benzyl ammonium chloride (55% C16, 20% C14); alkyl dimethyl benzyl ammonium chloride (58% C14, 28% C16); alkyl dimethyl benzyl ammonium chloride (60% C14, 25% C12); alkyl dimethyl benzyl ammonium chloride (61% C11, 23% C14); alkyl dimethyl benzyl ammonium chloride (61% C12, 23% C14); alkyl dimethyl benzyl ammonium chloride (65% C12, 25% C14); alkyl dimethyl benzyl ammonium chloride (67% C12, 24% C14); alkyl dimethyl benzyl ammonium chloride (67% C12, 25% C14); alkyl dimethyl benzyl ammonium chloride (90% C14, 5% C12); alkyl dimethyl benzyl ammonium chloride (93% C14, 4% C12); alkyl dimethyl benzyl ammonium chloride (95% C16, 5% C18); alkyl dimethyl benzyl ammonium chloride (and) didecyl dimethyl ammonium chloride; alkyl dimethyl benzyl ammonium chloride (as in fatty acids); alkyl dimethyl benzyl ammonium chloride (C12-C16); alkyl dimethyl benzyl ammonium chloride (C12-C18); alkyl dimethyl benzyl and dialkyl dimethyl ammonium chloride; alkyl dimethyl dimethylbenzyl ammonium chloride; alkyl dimethyl ethyl ammonium bromide (90% C14, 5% C16, 5% C12); alkyl dimethyl ethyl ammonium bromide (mixed alkyl and alkenyl groups as in the fatty acids of soybean oil); alkyl dimethyl ethylbenzyl ammonium chloride; alkyl dimethyl ethylbenzyl ammonium chloride (60% C14); alkyl dimethyl isopropylbenzyl ammonium chloride (50% C12, 30% C14, 17% C16, 3% C18); alkyl trimethyl ammonium chloride (58% C18, 40% C16, 1% C14, 1% C12); alkyl trimethyl ammonium chloride (90% C18, 10% C16); alkyl dimethyl(ethylbenzyl) ammonium chloride (C12-18); Di-(C8-10)-alkyl dimethyl ammonium chlorides; dialkyl dimethyl ammonium chloride; dialkyl dimethyl ammonium chloride; dialkyl methyl benzyl ammonium chloride; didecyl dimethyl ammonium chloride; diisodecyl dimethyl ammonium chloride; dioctyl dimethyl ammonium chloride; dodecyl bis (2-hydroxyethyl) octyl hydrogen ammonium chloride; dodecyl dimethyl benzyl ammonium chloride; dodecylcarbamoyl methyl dinethyl benzyl ammonium chloride; heptadecyl hydroxyethylimidazolinium chloride; hexahydro-

1,3,5-thris(2-hydroxyethyl)-s-triazine; myristalkonium chloride (and) Quat RNIUM 14; N,N-Dimethyl-2-hydroxypropylammonium chloride polymer; n-alkyl dimethyl benzyl ammonium chloride; n-alkyl dimethyl ethylbenzyl ammonium chloride; n-tetradecyl dimethyl benzyl ammonium chloride monohydrate; octyl decyl dimethyl ammonium chloride; octyl dodecyl dimethyl ammonium chloride; octyphenoxyethoxyethyl dimethyl benzyl ammonium chloride; oxydiethylenebis (alkyl dimethyl ammonium chloride); quaternary ammonium compounds, dicoco alkyldimethyl, chloride; trimethoxysilyl propyl dimethyl octadecyl ammonium chloride; trimethoxysilyl quats, trimethyl dodecylbenzyl ammonium chloride; n-dodecyl dimethyl ethylbenzyl ammonium chloride; n-hexadecyl dimethyl benzyl ammonium chloride; n-tetradecyl dimethyl benzyl ammonium chloride; n-tetradecyl dimethyl ethylbenzyl ammonium chloride; and n-octadecyl dimethyl benzyl ammonium chloride.

In general, the preferred non-toxic nanoemulsions are characterized by the following: they are approximately 200-800 nm in diameter, although both larger and smaller diameter nanoemulsions are contemplated; the charge depends on the ingredients; they are stable for relatively long periods of time (e.g., up to two years), with preservation of their biocidal activity; they are non-irritant and non-toxic compared to their individual components due, at least in part, to their oil contents that markedly reduce the toxicity of the detergents and the solvents; they are effective at concentrations as low as 0.1%; they have antimicrobial activity against most vegetative bacteria (including Gram-positive and Gram-negative organisms), fungi, and enveloped and nonenveloped viruses in 15 minutes (e.g., 99.99% killing); and they have sporicidal activity in 1-4 hours (e.g., 99.99% killing) when produced with germination enhancers.

D. Animal Models

In some embodiments, potential nanoemulsion compositions (e.g., for generating an immune response (e.g., for use as a vaccine) are tested in animal models of infectious diseases. The use of well-developed animal models provides a method of measuring the effectiveness and safety of a vaccine before administration to human subjects. Exemplary animal models of disease are shown in Table 3. These animals are commercially available (e.g., from Jackson Laboratories Charles River; Portage, MI).

Animal models of *Bacillus cereus* (closely related to *Bacillus anthracis*) are utilized to test Anthrax vaccines of the present invention. Both bacteria are spore forming Gram

positive rods and the disease syndrome produced by each bacteria is largely due to toxin production and the effects of these toxins on the infected host (Brown *et al.*, J. Bact., 75:499 (1958); Burdon and Wende, J. Infect Dis., 107:224 (1960); Burdon *et al.*, J. Infect. Dis., 117:307 (1967)). *Bacillus cereus* infection mimics the disease syndrome caused by *Bacillus anthracis*. Mice are reported to rapidly succumb to the effects of *B. cereus* toxin and are a useful model for acute infection. Guinea pigs develop a skin lesion subsequent to subcutaneous infection with *B. cereus* that resembles the cutaneous form of anthrax.

Clostridium perfringens infection in both mice and guinea pigs has been used as a model system for the *in vivo* testing of antibiotic drugs (Stevens *et al.*, Antimicrob. Agents Chemother., 31:312 (1987); Stevens *et al.*, J. Infect. Dis., 155:220 (1987); Altmeier *et al.*, Surgery, 28:621 (1950); Sandusky *et al.*, Surgery, 28:632 (1950)). *Clostridium tetani* is well known to infect and cause disease in a variety of mammalian species. Mice, guinea pigs, and rabbits have all been used experimentally (Willis, Topley and Wilson's Principles of Bacteriology, Virology and Immunity. Wilson, G., A. Miles, and M.T. Parker, eds. pages 442-475 1983).

Vibrio cholerae infection has been successfully initiated in mice, guinea pigs, and rabbits. According to published reports it is preferred to alter the normal intestinal bacterial flora for the infection to be established in these experimental hosts. This is accomplished by administration of antibiotics to suppress the normal intestinal flora and, in some cases, withholding food from the animals (Butterton *et al.*, Infect. Immun., 64:4373 (1996); Levine *et al.*, Microbiol. Rev., 47:510 (1983); Finkelstein *et al.*, J. Infect. Dis., 114:203 (1964); Freter, J. Exp. Med., 104:411 (1956); and Freter, J. Infect. Dis., 97:57 (1955)).

Shigella flexnerii infection has been successfully initiated in mice and guinea pigs. As is the case with vibrio infections, it is preferred that the normal intestinal bacterial flora be altered to aid in the establishment of infection in these experimental hosts. This is accomplished by administration of antibiotics to suppress the normal intestinal flora and, in some cases, withholding food from the animals (Levine *et al.*, Microbiol. Rev., 47:510 (1983); Freter, J. Exp. Med., 104:411 (1956); Formal *et al.*, J. Bact., 85:119 (1963); LaBrec *et al.*, J. Bact. 88:1503 (1964); Takeuchi *et al.*, Am. J. Pathol., 47:1011 (1965)).

Mice and rats have been used extensively in experimental studies with *Salmonella typhimurium* and *Salmonella enteritidis* (Naughton *et al.*, J. Appl. Bact., 81:651 (1996); Carter and Collins, J. Exp. Med., 139:1189 (1974); Collins, Infect. Immun., 5:191 (1972); Collins and Carter, Infect. Immun., 6:451 (1972)).

Mice and rats are well established experimental models for infection with *Sendai* virus (Jacoby *et al.*, Exp. Gerontol., 29:89 (1994); Massion *et al.*, Am. J. Respir. Cell Mol. Biol. 9:361 (1993); Castleman *et al.*, Am. J. Path., 129:277 (1987); Castleman, Am. J. Vet. Res., 44:1024 (1983); Mims and Murphy, Am. J. Path., 70:315 (1973)).

Sindbis virus infection of mice is usually accomplished by intracerebral inoculation of newborn mice. Alternatively, weanling mice are inoculated subcutaneously in the footpad (Johnson *et al.*, J. Infect. Dis., 125:257 (1972); Johnson, Am. J. Path., 46:929 (1965)).

It is preferred that animals are housed for 3-5 days to rest from shipping and adapt to new housing environments before use in experiments. At the start of each experiment, control animals are sacrificed and tissue is harvested to establish baseline parameters. Animals are anesthetized by any suitable method (e.g., including, but not limited to, inhalation of Isoflurane for short procedures or ketamine/xylazine injection for longer procedure).

Table 3 Animal Models of Infectious Diseases					
Microorganism	Experimental Animal Species	Experimental Animal Strains	Sex	Age	Route of Infection
<i>Francisella philomiraga</i>	mice	BALB/C	M	6 W	Intraperitoneal
<i>Neisseria meningitidis</i>	mice	BALB/C	F	6-10 W	Intraperitoneal
	rats	COBS/CD	M/F	4 D	Intranasal
<i>Streptococcus pneumoniae</i>	mice	BALB/C	F	6 W	Intranasal
	rats	COBS/CD	M	6-8 W	Intranasal
	guinea Pigs	Hartley	M/F	4-5 W	Intranasal

<i>Yersinia pseudotuberculosis</i>	mice	BALB/C	F	6 W	Intranasal
<i>Influenza virus</i>	mice	BALB/C	F	6 W	Intranasal
<i>Sendai virus</i>	mice	CD-1	F	6 W	Intranasal
	rats	Sprague-Dawley	M	6-8 W	Intranasal
<i>Sindbis</i>	mice	CD-1	M/F	1-2 D	Intracerebral/SC
<i>Vaccinia</i>	mice	BALB/C	F	2-3 W	Intradermal

E. Assays For Evaluation of Vaccines

In some embodiments, candidate nanoemulsion vaccines are evaluated using one of several suitable model systems. For example, cell-mediated immune responses can be evaluated *in vitro*. In addition, an animal model may be used to evaluate *in vivo* immune response and immunity to pathogen challenge. Any suitable animal model may be utilized, including, but not limited to, those disclosed in Table 3.

Before testing a nanoemulsion vaccine in an animal system, the amount of exposure of the pathogen to a nanoemulsion sufficient to inactivate the pathogen is investigated. It is contemplated that pathogens such as bacterial spores require longer periods of time for inactivation by the nanoemulsion in order to be sufficiently neutralized to allow for immunization. The time period required for inactivation may be investigated using any suitable method, including, but not limited to, those described in the illustrative examples below.

In addition, the stability of emulsion-developed vaccines is evaluated, particularly over time and storage condition, to ensure that vaccines are effective long-term. The ability of other stabilizing materials (*e.g.*, dendritic polymers) to enhance the stability and immunogenicity of vaccines is also evaluated.

Once a given nanoemulsion/pathogen vaccine has been formulated to result in pathogen inactivation, the ability of the vaccine to elicit an immune response and provide immunity is optimized. Non-limiting examples of methods for assaying vaccine effectiveness are described in Example 14 below. For example, the timing and dosage of

the vaccine can be varied and the most effective dosage and administration schedule determined. The level of immune response is quantitated by measuring serum antibody levels. In addition, *in vitro* assays are used to monitor proliferation activity by measuring H^3 -thymidine uptake. In addition to proliferation, Th1 and Th2 cytokine responses (*e.g.*, including but not limited to, levels of include IL-2, TNF- γ , IFN- γ , IL-4, IL-6, IL-11, IL-12, etc.) are measured to qualitatively evaluate the immune response.

Finally, animal models are utilized to evaluate the effect of a nanoemulsion mucosal vaccine. Purified pathogens are mixed in emulsions (or emulsions are contact with a pre-infected animal), administered, and the immune response is determined. The level of protection is then evaluated by challenging the animal with the specific pathogen and subsequently evaluating the level of disease symptoms. The level of immunity is measured over time to determine the necessity and spacing of booster immunizations.

III. Therapeutics and Prophylactics

Furthermore, in preferred embodiments, a composition of the present invention induces (*e.g.*, when administered to a subject) both systemic and mucosal immunity. Thus, in some preferred embodiments, administration of a composition of the present invention to a subject results in protection against an exposure (*e.g.*, a mucosal exposure) to HIV. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, mucosal administration (*e.g.*, vaccination) provides protection against HIV infection (*e.g.*, that initiates at a mucosal surface). Although it has heretofore proven difficult to stimulate secretory IgA responses and protection against pathogens that invade at mucosal surfaces (See, *e.g.*, Mestecky et al, *Mucosal Immunology*. 3ed edn. (Academic Press, San Diego, 2005)), the present invention provides compositions and methods for stimulating mucosal immunity (*e.g.*, a protective IgA response) from a pathogen in a subject.

In some embodiments, the present invention provides a composition (*e.g.*, a composition comprising a NE and immunogenic protein antigens from HIV (*e.g.*, gp120) to serve as a mucosal vaccine. This material can easily be produced with NE and HIV protein (*e.g.*, viral-derived gp120, live-virus-vector-derived gp120 and gp160, recombinant mammalian gp120, recombinant denatured antigens, small peptide segments of gp120 and gp41, V3 loop peptides, and induces both mucosal and systemic immunity. The ability to produce this formulation rapidly and administer it via mucosal (*e.g.*, nasal or vaginal)

instillation provides a vaccine that can be used in large-scale administrations (e.g., to a population of a town, village, city, state or country).

In some preferred embodiments, the present invention provides a composition for generating an immune response comprising a NE and an immunogen (e.g., a purified, isolated or synthetic HIV protein or derivative, variant, or analogue thereof; or, one or more serotypes of HIV inactivated by the nanoemulsion). When administered to a subject, a composition of the present invention stimulates an immune response against the immunogen within the subject. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, generation of an immune response (e.g., resulting from administration of a composition comprising a nanoemulsion and an immunogen) provides total or partial immunity to the subject (e.g., from signs, symptoms or conditions of a disease (e.g., AIDS)). Without being bound to any specific theory, protection and/or immunity from disease (e.g., the ability of a subject's immune system to prevent or attenuate (e.g., suppress) a sign, symptom or condition of disease) after exposure to an immunogenic composition of the present invention is due to adaptive (e.g., acquired) immune responses (e.g., immune responses mediated by B and T cells following exposure to a NE comprising an immunogen of the present invention (e.g., immune responses that exhibit increased specificity and reactivity towards HIV)). Thus, in some embodiments, the compositions and methods of the present invention are used prophylactically or therapeutically to prevent or attenuate a sign, symptom or condition associated with AIDS.

In some embodiments, a NE comprising an immunogen (e.g., a recombinant HIV protein) is administered alone. In some embodiments, a composition comprising a NE and an immunogen (e.g., a recombinant HIV protein) comprises one or more other agents (e.g., a pharmaceutically acceptable carrier, adjuvant, excipient, and the like). In some embodiments, a composition for stimulating an immune response of the present invention is administered in a manner to induce a humoral immune response. In some embodiments, a composition for stimulating an immune response of the present invention is administered in a manner to induce a cellular (e.g., cytotoxic T lymphocyte) immune response, rather than a humoral response. In some embodiments, a composition comprising a NE and an immunogen of the present invention induces both a cellular and humoral immune response.

The present invention is not limited by the type or strain of orthopox virus used (e.g., in a composition comprising a NE and immunogen (e.g., orthopox virus inactivated by the

nanoemulsion). Indeed, each orthopox virus family member alone, or in combination with another family member, may be used to generate a composition comprising a NE and an immunogen (e.g., used to generate an immune response) of the present invention. Orthopox virus family member include, but are not limited to, variola virus, vaccinia virus, cowpox, monkeypox, gergilpox, camelpox, and others. The present invention is not limited by the strain of vaccinia virus used. Indeed, a variety of vaccinia virus strains are contemplated to be useful in the present invention including, but not limited to, classical strains of vaccinia virus (e.g., EM-63, Lister, New York City Board of Health, Elestree, and Temple of Heaven strains), attenuated strains (e.g., Ankara), non-replicating strains, modified strains (e.g., genetically or mechanically modified strains (e.g., to become more or less virulent)), Copenhagen strain, modified vaccinia Ankara, New York vaccinia virus, Vaccinia Virus_{WR} and Vaccinia Virus_{WR-Luc}, or other serially diluted strain of vaccinia virus. A composition comprising a NE and immunogen may comprise one or more strains of vaccinia virus and/or other type of orthopox virus. Additionally, a composition comprising a NE and immunogen may comprise one or more strains of vaccinia virus, and, in addition, one or more strains of a non-vaccinia virus immunogen or immunogenic epitope thereof (e.g., a bacteria (e.g., *B. anthracis*) or immunogenic epitope thereof (e.g., recombinant protective antigen) or a virus (e.g., West Nile virus, Avian Influenza virus, Ebola virus, HSV, HPV, HCV, HIV, etc.) or an immunogenic epitope thereof (e.g., gp120)).

In some embodiments, the immunogen may comprise one or more antigens derived from a pathogen (e.g., orthopox virus). For example, in some embodiments, the immunogen is a purified, recombinant, synthetic, or otherwise isolated protein (e.g., added to the NE to generate an immunogenic composition). Similarly, the immunogenic protein may be a derivative, analogue or otherwise modified (e.g., PEGylated) form of a protein from a pathogen.

The present invention is not limited by the type or strain of *Bacillus* used or immunogenic protein derived therefrom. For example, 89 different strains of *B. anthracis* have been identified, ranging from virulent Ames and *Vollum* strains with biological warfare and bioterrorism applications to benign *Sterne* strain used for inoculations (See, e.g.,

Easterday et al., J Clin Microbiol. 2005 43(4):1995-7). The strains differ in presence and activity of various genes, determining their virulence and production of antigens and toxins.

Any one of these or yet to be identified or generated strains may be used in an immunogenic composition comprising a NE of the present invention.

In some embodiments, the immunogen may comprise one or more antigens derived from a pathogen (e.g., *B. anthracis*). For example, in some embodiments, the immunogen is a purified, recombinant, synthetic, or otherwise isolated protein (e.g., added to the NE to generate an immunogenic composition). Similarly, the immunogenic protein may be a derivative, variant, analogue or otherwise modified form of a protein from a pathogen. The present invention is not limited by the type of protein (e.g., derived from bacteria of the genus *Bacillus*) used for generation of an immunogenic composition of the present invention. Indeed, a variety of immunogenic proteins may be used including, but not limited to, protective antigen (PA), lethal factor (LF), edema factor (EF), PA degradation products (See, e.g., Farchaus, J., et al., *Applied & Environmental Microbiol.*, 64(3):982-991 (1998)), as well as analogues, derivatives and modified forms thereof.

For example, *Bacillus* proteins of the present invention may be used in their native conformation, or more preferably, may be modified for vaccine use. These modifications may either be required for technical reasons relating to the method of purification, or they may be used to biologically inactivate one or several functional properties of the *Bacillus* proteins (e.g., that would otherwise be toxic). Thus the invention encompasses derivatives of *Bacillus* proteins that may be, for example, mutated proteins (e.g., that has undergone deletion, addition or substitution of one or more amino acids using well known techniques for site directed mutagenesis or any other conventional method).

Bacillus proteins (e.g., rPA) of the present invention may be modified by chemical methods during a purification process to render the proteins stable and monomeric. One method to prevent oxidative aggregation of a protein is the use of chemical modifications of the protein's thiol groups. In a first step the disulphide bridges are reduced by treatment with a reducing agent such as DTT, β -mercaptoethanol, or glutathione. In a second step the resulting thiols are blocked by reaction with an alkylating agent (e.g., the protein can be carboxyamidated/carbamidomethylated using iodoacetamide).

Each *Bacillus* family member alone, or in combination with another family member, may be used to generate a composition comprising a NE and an immunogen (e.g., used to generate an immune response) of the present invention. A composition comprising a NE and immunogen may comprise one or more strains of *B. anthracis*. Additionally, a composition comprising a NE and immunogen may comprise one or more strains of *B. anthracis*, and, in addition, one or more strains of a non-*B. anthracis* immunogen (e.g., a virus such as West Nile virus, Avian Influenza virus, Ebola virus, HSV, HPV, HCV, HIV,

etc. or an immunogenic epitope thereof (e.g., gp120)).

The present invention is not limited by the type (e.g., serotype, group, or clade) of HIV used or immunogenic protein derived therefrom. For example, there are currently two types of HIV: HIV-1 and HIV-2. Both types are transmitted by sexual contact, through blood, and from mother to child, and they appear to cause clinically indistinguishable AIDS. However, it seems that HIV-2 is less easily transmitted, and the period between initial infection and illness is longer in the case of HIV-2. Worldwide, the predominant virus is HIV-1, and generally when people refer to HIV without specifying the type of virus they will be referring to HIV-1. The relatively uncommon HIV-2 type is concentrated in West Africa and is rarely found elsewhere.

Different levels of HIV classification exist. Each type is divided into groups, and each group is divided into subtypes and circulating recombinant forms (CRFs). The strains of HIV-1 can be classified into three groups : the "major" group M, the "outlier" group O and the "new" group N.

Within group M there are known to be at least nine genetically distinct subtypes (or clades) of HIV-1. These are subtypes A, B, C, D, F, G, H, J and K.

Any one of these or yet to be identified or generated serotypes, groups, or clades may be used in an immunogenic composition comprising a NE of the present invention.

In some embodiments, the immunogen may comprise one or more antigens derived from a pathogen (e.g., HIV). For example, in some embodiments, the immunogen is a purified, recombinant, synthetic, or otherwise isolated protein (e.g., added to the NE to generate an immunogenic composition). Similarly, the immunogenic protein may be a derivative, analogue or otherwise modified form of a protein from a pathogen. The present invention is not limited by the type of protein (e.g., derived from HIV) used for generation of an immunogenic composition of the present invention. Indeed, a variety of immunogenic proteins may be used including, but not limited to, gp160, gp120, gp41, Tat, and Nef; as well as analogues, derivatives and modified forms thereof.

For example, HIV proteins of the present invention may be used in their native conformation, or more preferably, may be modified for vaccine use. These modifications may either be required for technical reasons relating to the method of purification, or they may be used to biologically inactivate one or several functional properties of HIV protein. Thus the invention encompasses derivatives of HIV proteins which may be, for example mutated proteins (e.g., that has undergone deletion, addition or substitution of one or more

amino acids using well known techniques for site directed mutagenesis or any other conventional method.

For example, a HIV protein may be mutated so that it is biologically inactive while maintaining its immunogenic epitopes (See, e.g., Clements, Virology 235: 48-64, 1997).

Additionally, HIV proteins of the present invention may be modified by chemical methods during the purification process to render the proteins stable and monomeric. One method to prevent oxidative aggregation of a HIV protein is the use of chemical modifications of the protein's thiol groups. In a first step the disulphide bridges are reduced by treatment with a reducing agent such as DTT, β -mercaptoethanol, or glutathione. In a second step the resulting thiols are blocked by reaction with an alkylating agent (e.g., the protein can be carboxyamidated/carbamidomethylated using iodoacetamide).

Each HIV serotype, group or clade alone, or in combination with another family member, may be used to generate a composition comprising a NE and an immunogen (e.g., used to generate an immune response) of the present invention. A composition comprising a NE and immunogen may comprise one or more serotypes, groups or clades of HIV. Additionally, a composition comprising a NE and immunogen may comprise one or more serotypes, groups or clades of HIV, and, in addition, one or more strains of a non-HIV immunogen (e.g., a virus such as West Nile virus, Avian Influenza virus, Ebola virus, HSV, HPV, HCV, , etc. or an immunogenic epitope thereof).

The present invention is not limited by the particular formulation of a composition comprising a NE and immunogen of the present invention. Indeed, a composition comprising a NE and immunogen of the present invention may comprise one or more different agents in addition to the NE and immunogen. These agents or cofactors include, but are not limited to, adjuvants, surfactants, additives, buffers, solubilizers, chelators, oils, salts, therapeutic agents, drugs, bioactive agents, antibacterials, and antimicrobial agents (e.g., antibiotics, antivirals, etc.). In some embodiments, a composition comprising a NE and immunogen of the present invention comprises an agent and/or co-factor that enhance the ability of the immunogen to induce an immune response (e.g., an adjuvant). In some preferred embodiments, the presence of one or more co-factors or agents reduces the amount of immunogen required for induction of an immune response (e.g., a protective immune response (e.g., protective immunization)). In some embodiments, the presence of one or more co-factors or agents can be used to skew the immune response towards a cellular (e.g., T cell mediated) or humoral (e.g., antibody mediated) immune response. The present

invention is not limited by the type of co-factor or agent used in a therapeutic agent of the present invention.

Adjuvants are described in general in *Vaccine Design--the Subunit and Adjuvant Approach*, edited by Powell and Newman; Plenum Press, New York, 1995. The present invention is not limited by the type of adjuvant utilized (e.g., for use in a composition (e.g., pharmaceutical composition) comprising a NE and immunogen). For example, in some embodiments, suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate. In some embodiments, an adjuvant may be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

In some embodiments, it is preferred that a composition comprising a NE and immunogen of the present invention comprises one or more adjuvants that induce a Th1-type response. However, in other embodiments, it will be preferred that a composition comprising a NE and immunogen of the present invention comprises one or more adjuvants that induce a Th2-type response.

In general, an immune response is generated to an antigen through the interaction of the antigen with the cells of the immune system. Immune responses may be broadly categorized into two categories: humoral and cell mediated immune responses (e.g., traditionally characterized by antibody and cellular effector mechanisms of protection, respectively). These categories of response have been termed Th1-type responses (cell-mediated response), and Th2-type immune responses (humoral response).

Stimulation of an immune response can result from a direct or indirect response of a cell or component of the immune system to an intervention (e.g., exposure to an immunogen). Immune responses can be measured in many ways including activation, proliferation or differentiation of cells of the immune system (e.g., B cells, T cells, dendritic cells, APCs, macrophages, NK cells, NKT cells etc.); up-regulated or down-regulated expression of markers and cytokines; stimulation of IgA, IgM, or IgG titer; splenomegaly (including increased spleen cellularity); hyperplasia and mixed cellular infiltrates in various organs. Other responses, cells, and components of the immune system that can be assessed with respect to immune stimulation are known in the art.

Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, compositions and methods of the present invention induce expression

and secretion of cytokines (e.g., by macrophages, dendritic cells and CD4+ T cells). Modulation of expression of a particular cytokine can occur locally or systemically. It is known that cytokine profiles can determine T cell regulatory and effector functions in immune responses. In some embodiments, Th1-type cytokines can be induced, and thus, the immunostimulatory compositions of the present invention can promote a Th1 type antigen-specific immune response including cytotoxic T-cells. However in other embodiments, Th2-type cytokines can be induced thereby promoting a Th2 type antigen-specific immune response.

Cytokines play a role in directing the T cell response. Helper (CD4+) T cells orchestrate the immune response of mammals through production of soluble factors that act on other immune system cells, including B and other T cells. Most mature CD4+T helper cells express one of two cytokine profiles: Th1 or Th2. Th1-type CD4+ T cells secrete IL-2, IL-3, IFN- γ , GM-CSF and high levels of TNF- α . Th2 cells express IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF and low levels of TNF- α . Th1 type cytokines promote both cell-mediated immunity, and humoral immunity that is characterized by immunoglobulin class switching to IgG2a in mice and IgG1 in humans. Th1 responses may also be associated with delayed-type hypersensitivity and autoimmune disease. Th2 type cytokines induce primarily humoral immunity and induce class switching to IgG1 and IgE. The antibody isotypes associated with Th1 responses generally have neutralizing and opsonizing capabilities whereas those associated with Th2 responses are associated more with allergic responses.

Several factors have been shown to influence skewing of an immune response towards either a Th1 or Th2 type response. The best characterized regulators are cytokines. IL-12 and IFN- γ are positive Th1 and negative Th2 regulators. IL-12 promotes IFN- γ production, and IFN- γ provides positive feedback for IL-12. IL-4 and IL-10 appear important for the establishment of the Th2 cytokine profile and to down-regulate Th1 cytokine production.

Thus, in some preferred embodiments, the present invention provides a method of stimulating a Th1-type immune response in a subject comprising administering to a subject a composition comprising a NE and an immunogen. However, in other preferred embodiments, the present invention provides a method of stimulating a Th2-type immune response in a subject comprising administering to a subject a composition comprising a NE and an immunogen. In further preferred embodiments, adjuvants can be used (e.g., can be

co-administered with a composition of the present invention) to skew an immune response toward either a Th1 or Th2 type immune response. For example, adjuvants that induce Th2 or weak Th1 responses include, but are not limited to, alum, saponins, and SB-As4. Adjuvants that induce Th1 responses include but are not limited to MPL, MDP, ISCOMS, IL-12, IFN- γ , and SB-AS2.

Several other types of Th1-type immunogens can be used (e.g., as an adjuvant) in compositions and methods of the present invention. These include, but are not limited to, the following. In some embodiments, monophosphoryl lipid A (e.g., in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL)), is used. 3D-MPL is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid A with either 4, 5, or 6 acylated chains. In some embodiments, diphosphoryl lipid A, and 3-O-deacylated variants thereof are used. Each of these immunogens can be purified and prepared by methods described in GB 2122204B, hereby incorporated by reference in its entirety. Other purified and synthetic lipopolysaccharides have been described (See, e.g., U.S. Pat. No. 6,005,099 and EP 0 729 473; Hilgers et al., 1986, *Int.Arch.Allergy.Immunol.*, 79(4):392-6; Hilgers et al., 1987, *Immunology*, 60(1):141-6; and EP 0 549 074, each of which is hereby incorporated by reference in its entirety). In some embodiments, 3D-MPL is used in the form of a particulate formulation (e.g., having a small particle size less than 0.2 μm in diameter, described in EP 0 689 454, hereby incorporated by reference in its entirety).

In some embodiments, saponins are used as an immunogen (e.g., Th1-type adjuvant) in a composition of the present invention. Saponins are well known adjuvants (See, e.g., Lacaille-Dubois and Wagner (1996) *Phytomedicine* vol 2 pp 363-386). Examples of saponins include Quil A (derived from the bark of the South American tree *Quillaja Saponaria Molina*), and fractions thereof (See, e.g., U.S. Pat. No. 5,057,540; Kensil, *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279, each of which is hereby incorporated by reference in its entirety). Also contemplated to be useful in the present invention are the haemolytic saponins QS7, QS17, and QS21 (HPLC purified fractions of Quil A; See, e.g., Kensil et al. (1991). *J. Immunology* 146,431-437, U.S. Pat. No. 5,057,540; WO 96/33739; WO 96/11711 and EP 0 362 279, each of which is hereby incorporated by reference in its entirety). Also contemplated to be useful are combinations of QS21 and polysorbate or cyclodextrin (See, e.g., WO 99/10008, hereby incorporated by reference in its entirety).

In some embodiments, an immunogenic oligonucleotide containing unmethylated CpG dinucleotides ("CpG") is used as an adjuvant in the present invention. CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (See, e.g., WO 96/02555, EP 468520, Davis et al., J.Immunol, 1998, 160(2):870-876; McCluskie and Davis, J.Immunol., 1998, 161(9):4463-6; and U.S. Pat. App. No. 20050238660, each of which is hereby incorporated by reference in its entirety). For example, in some embodiments, the immunostimulatory sequence is Purine-Purine-C-G-pyrimidine-pyrimidine; wherein the CG motif is not methylated.

Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, the presence of one or more CpG oligonucleotides activate various immune subsets including natural killer cells (which produce IFN- γ) and macrophages. In some embodiments, CpG oligonucleotides are formulated into a composition of the present invention for inducing an immune response. In some embodiments, a free solution of CpG is co-administered together with an antigen (e.g., present within a NE solution (See, e.g., WO 96/02555; hereby incorporated by reference). In some embodiments, a CpG oligonucleotide is covalently conjugated to an antigen (See, e.g., WO 98/16247, hereby incorporated by reference), or formulated with a carrier such as aluminium hydroxide (See, e.g., Brazolot-Millan et al., Proc.Natl.AcadSci., USA, 1998, 95(26), 15553-8).

In some embodiments, adjuvants such as Complete Freund's Adjuvant and Incomplete Freund's Adjuvant, cytokines (e.g., interleukins (e.g., IL-2, IFN- γ , IL-4, etc.), macrophage colony stimulating factor, tumor necrosis factor, etc.), detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an E. Coli heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (See, e.g., WO93/13202 and WO92/19265, each of which is hereby incorporated by reference), and other immunogenic substances (e.g., that enhance the effectiveness of a composition of the present invention) are used with a composition comprising a NE and immunogen of the present invention.

Additional examples of adjuvants that find use in the present invention include

poly(di(carboxylatophenoxy)phosphazene (PCPP polymer; Virus Research Institute, USA); derivatives of lipopolysaccharides such as monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Inc., Hamilton, Mont.), muramyl dipeptide (MDP; Ribi) and threonyl-muramyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland); and Leishmania elongation factor (a purified Leishmania protein; Corixa Corporation, Seattle, Wash.).

Adjuvants may be added to a composition comprising a NE and an immunogen, or, the adjuvant may be formulated with carriers, for example liposomes, or metallic salts (e.g., aluminium salts (e.g., aluminium hydroxide)) prior to combining with or co-administration with a composition comprising a NE and an immunogen.

In some embodiments, a composition comprising a NE and an immunogen comprises a single adjuvant. In other embodiments, a composition comprising a NE and an immunogen comprises two or more adjuvants (See, e.g., WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241; and WO 94/00153, each of which is hereby incorporated by reference in its entirety).

In some embodiments, a composition comprising a NE and an immunogen of the present invention comprises one or more mucoadhesives (See, e.g., U.S. Pat. App. No. 20050281843, hereby incorporated by reference in its entirety). The present invention is not limited by the type of mucoadhesive utilized. Indeed, a variety of mucoadhesives are contemplated to be useful in the present invention including, but not limited to, cross-linked derivatives of poly(acrylic acid) (e.g., carbopol and polycarbophil), polyvinyl alcohol, polyvinyl pyrrolidone, polysaccharides (e.g., alginate and chitosan), hydroxypropyl methylcellulose, lectins, fimbrial proteins, and carboxymethylcellulose. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, use of a mucoadhesive (e.g., in a composition comprising a NE and immunogen) enhances induction of an immune response in a subject (e.g., administered a composition of the present invention) due to an increase in duration and/or amount of exposure to an immunogen that a subject experiences when a mucoadhesive is used compared to the duration and/or amount of exposure to an immunogen in the absence of using the mucoadhesive.

In some embodiments, a composition of the present invention may comprise sterile aqueous preparations. Acceptable vehicles and solvents include, but are not limited to,

water, Ringer's solution, phosphate buffered saline and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed mineral or non-mineral oil may be employed including synthetic mono-ordi-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for mucosal, subcutaneous, intramuscular, intraperitoneal, intravenous, or administration via other routes may be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa.

A composition comprising a NE and an immunogen of the present invention can be used therapeutically (e.g., to enhance an immune response) or as a prophylactic (e.g., for immunization (e.g., to prevent signs or symptoms of disease)). A composition comprising a NE and an immunogen of the present invention can be administered to a subject via a number of different delivery routes and methods.

For example, the compositions of the present invention can be administered to a subject (e.g., mucosally (e.g., nasal mucosa, vaginal mucosa, etc.)) by multiple methods, including, but not limited to: being suspended in a solution and applied to a surface; being suspended in a solution and sprayed onto a surface using a spray applicator; being mixed with a mucoadhesive and applied (e.g., sprayed or wiped) onto a surface (e.g., mucosal surface); being placed on or impregnated onto a nasal and/or vaginal applicator and applied; being applied by a controlled-release mechanism; being applied as a liposome; or being applied on a polymer.

In some preferred embodiments, compositions of the present invention are administered mucosally (e.g., using standard techniques; See, e.g., Remington: The Science and Practice of Pharmacy, Mack Publishing Company, Easton, Pa., 19th edition, 1995 (e.g., for mucosal delivery techniques, including intranasal, pulmonary, vaginal and rectal techniques), as well as European Publication No. 517,565 and Illum et al., J. Controlled Rel., 1994, 29:133-141 (e.g., for techniques of intranasal administration), each of which is hereby incorporated by reference in its entirety). Alternatively, the compositions of the present invention may be administered dermally or transdermally, using standard techniques (See, e.g., Remington: The Science and Practice of Pharmacy, Mack Publishing Company, Easton, Pa., 19th edition, 1995). The present invention is not limited by the route of administration.

Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in

some embodiments, mucosal vaccination is the preferred route of administration as it has been shown that mucosal administration of antigens has a greater efficacy of inducing protective immune responses at mucosal surfaces (e.g., mucosal immunity), the route of entry of many pathogens. In addition, mucosal vaccination, such as intranasal vaccination, may induce mucosal immunity not only in the nasal mucosa, but also in distant mucosal sites such as the genital mucosa (See, e.g., Mestecky, Journal of Clinical Immunology, 7:265-276, 1987). More advantageously, in further preferred embodiments, in addition to inducing mucosal immune responses, mucosal vaccination also induces systemic immunity. In some embodiments, non-parenteral administration (e.g., mucosal administration of vaccines) provides an efficient and convenient way to boost systemic immunity (e.g., induced by parenteral or mucosal vaccination (e.g., in cases where multiple boosts are used to sustain a vigorous systemic immunity)).

In some embodiments, a composition comprising a NE and an immunogen of the present invention may be used to protect or treat a subject susceptible to, or suffering from, disease by means of administering a composition of the present invention via a mucosal route (e.g., an oral/alimentary or nasal route). Alternative mucosal routes include intravaginal and intra-rectal routes. In preferred embodiments of the present invention, a nasal route of administration is used, termed "intranasal administration" or "intranasal vaccination" herein. Methods of intranasal vaccination are well known in the art, including the administration of a droplet or spray form of the vaccine into the nasopharynx of a subject to be immunized. In some embodiments, a nebulized or aerosolized composition comprising a NE and immunogen is provided. Enteric formulations such as gastro resistant capsules for oral administration, suppositories for rectal or vaginal administration also form part of this invention. Compositions of the present invention may also be administered via the oral route. Under these circumstances, a composition comprising a NE and an immunogen may comprise a pharmaceutically acceptable excipient and/or include alkaline buffers, or enteric capsules. Formulations for nasal delivery may include those with dextran or cyclodextran and saponin as an adjuvant.

Compositions of the present invention may also be administered via a vaginal route. In such cases, a composition comprising a NE and an immunogen may comprise pharmaceutically acceptable excipients and/or emulsifiers, polymers (e.g., CARBOPOL), and other known stabilizers of vaginal creams and suppositories. In some embodiments, compositions of the present invention are administered via a rectal route. In such cases, a

composition comprising a NE and an immunogen may comprise excipients and/or waxes and polymers known in the art for forming rectal suppositories.

In some embodiments, the same route of administration (e.g., mucosal administration) is chosen for both a priming and boosting vaccination. In some embodiments, multiple routes of administration are utilized (e.g., at the same time, or, alternatively, sequentially) in order to stimulate an immune response (e.g., using a composition comprising a NE and immunogen of the present invention).

For example, in some embodiments, a composition comprising a NE and an immunogen is administered to a mucosal surface of a subject in either a priming or boosting vaccination regime. Alternatively, in some embodiments, a composition comprising a NE and an immunogen is administered systemically in either a priming or boosting vaccination regime. In some embodiments, a composition comprising a NE and an immunogen is administered to a subject in a priming vaccination regimen via mucosal administration and a boosting regimen via systemic administration. In some embodiments, a composition comprising a NE and an immunogen is administered to a subject in a priming vaccination regimen via systemic administration and a boosting regimen via mucosal administration. Examples of systemic routes of administration include, but are not limited to, a parenteral, intramuscular, intradermal, transdermal, subcutaneous, intraperitoneal or intravenous administration. A composition comprising a NE and an immunogen may be used for both prophylactic and therapeutic purposes.

In some embodiments, compositions of the present invention are administered by pulmonary delivery. For example, a composition of the present invention can be delivered to the lungs of a subject (e.g., a human) via inhalation (e.g., thereby traversing across the lung epithelial lining to the blood stream (See, e.g., Adjei, et al. *Pharmaceutical Research* 1990; 7:565-569; Adjei, et al. *Int. J. Pharmaceutics* 1990; 63:135-144; Braquet, et al. *J. Cardiovascular Pharmacology* 1989 143-146; Hubbard, et al. (1989) *Annals of Internal Medicine*, Vol. III, pp. 206-212; Smith, et al. *J. Clin. Invest.* 1989;84:1145-1146; Oswein, et al. "Aerosolization of Proteins", 1990; *Proceedings of Symposium on Respiratory Drug Delivery II* Keystone, Colorado; Debs, et al. *J. Immunol.* 1988; 140:3482-3488; and U.S. Pat. No. 5,284,656 to Platz, et al, each of which are hereby incorporated by reference in its entirety). A method and composition for pulmonary delivery of drugs for systemic effect is described in U.S. Pat. No. 5,451,569 to Wong, et al., hereby incorporated by reference; See also U.S. Pat. No. 6,651,655 to Licalsi et al., hereby incorporated by reference in its

entirety)).

Further contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary and/or nasal mucosal delivery of pharmaceutical agents including, but not limited to, nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer (Mallinckrodt Inc., St. Louis, Mo.); the Acorn II nebulizer (Marquest Medical Products, Englewood, Colo.); the Ventolin metered dose inhaler (Glaxo Inc., Research Triangle Park, N.C.); and the Spinhaler powder inhaler (Fisons Corp., Bedford, Mass.). All such devices require the use of formulations suitable for dispensing of the therapeutic agent. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, adjuvants, surfactants, carriers and/or other agents useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated.

Thus, in some embodiments, a composition comprising a NE and an immunogen of the present invention may be used to protect and/or treat a subject susceptible to, or suffering from, a disease by means of administering a compositions comprising a NE and an immunogen by mucosal, intramuscular, intraperitoneal, intradermal, transdermal, pulmonary, intravenous, subcutaneous or other route of administration described herein. Methods of systemic administration of the vaccine preparations may include conventional syringes and needles, or devices designed for ballistic delivery of solid vaccines (See, e.g., WO 99/27961, hereby incorporated by reference), or needleless pressure liquid jet device (See, e.g., U.S. Pat. No. 4,596,556; U.S. Pat. No. 5,993,412, each of which are hereby incorporated by reference), or transdermal patches (See, e.g., WO 97/48440; WO 98/28037, each of which are hereby incorporated by reference). The present invention may also be used to enhance the immunogenicity of antigens applied to the skin (transdermal or transcutaneous delivery, See, e.g., WO 98/20734 ; WO 98/28037, each of which are hereby incorporated by reference). Thus, in some embodiments, the present invention provides a delivery device for systemic administration, pre-filled with the vaccine composition of the present invention.

The present invention is not limited by the type of subject administered (e.g., in order to stimulate an immune response (e.g., in order to generate protective immunity (e.g.,

mucosal and/or systemic immunity))) a composition of the present invention. Indeed, a wide variety of subjects are contemplated to be benefited from administration of a composition of the present invention. In preferred embodiments, the subject is a human. In some embodiments, human subjects are of any age (e.g., adults, children, infants, etc.) that have been or are likely to become exposed to a microorganism. In some embodiments, the human subjects are subjects that are more likely to receive a direct exposure to pathogenic microorganisms or that are more likely to display signs and symptoms of disease after exposure to a pathogen (e.g., immune suppressed subjects). In some embodiments, the general public is administered (e.g., vaccinated with) a composition of the present invention (e.g., to prevent the occurrence or spread of disease). For example, in some embodiments, compositions and methods of the present invention are utilized to vaccinate a group of people (e.g., a population of a region, city, state and/or country) for their own health (e.g., to prevent or treat disease). In some embodiments, the subjects are non-human mammals (e.g., pigs, cattle, goats, horses, sheep, or other livestock; or mice, rats, rabbits or other animal). In some embodiments, compositions and methods of the present invention are utilized in research settings (e.g., with research animals).

A composition of the present invention may be formulated for administration by any route, such as mucosal, oral, topical, parenteral or other route described herein. The compositions may be in any one or more different forms including, but not limited to, tablets, capsules, powders, granules, lozenges, foams, creams or liquid preparations.

Topical formulations of the present invention may be presented as, for instance, ointments, creams or lotions, foams, and aerosols, and may contain appropriate conventional additives such as preservatives, solvents (e.g., to assist penetration), and emollients in ointments and creams.

Topical formulations may also include agents that enhance penetration of the active ingredients through the skin. Exemplary agents include a binary combination of N-(hydroxyethyl) pyrrolidone and a cell-envelope disordering compound, a sugar ester in combination with a sulfoxide or phosphine oxide, and sucrose monooleate, decyl methyl sulfoxide, and alcohol.

Other exemplary materials that increase skin penetration include surfactants or wetting agents including, but not limited to, polyoxyethylene sorbitan mono-oleate (Polysorbate 80); sorbitan mono-oleate (Span 80); p-isooctyl polyoxyethylene-phenol polymer (Triton WR-1330); polyoxyethylene sorbitan tri-oleate (Tween 85); dioctyl sodium

sulfosuccinate; and sodium sarcosinate (Sarcosyl NL-97); and other pharmaceutically acceptable surfactants.

In certain embodiments of the invention, compositions may further comprise one or more alcohols, zinc-containing compounds, emollients, humectants, thickening and/or gelling agents, neutralizing agents, and surfactants. Water used in the formulations is preferably deionized water having a neutral pH. Additional additives in the topical formulations include, but are not limited to, silicone fluids, dyes, fragrances, pH adjusters, and vitamins.

Topical formulations may also contain compatible conventional carriers, such as cream or ointment bases and ethanol or oleyl alcohol for lotions. Such carriers may be present as from about 1% up to about 98% of the formulation. The ointment base can comprise one or more of petrolatum, mineral oil, ceresin, lanolin alcohol, panthenol, glycerin, bisabolol, cocoa butter and the like.

In some embodiments, pharmaceutical compositions of the present invention may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, preferably do not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents (e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like) that do not deleteriously interact with the NE and immunogen of the formulation. In some embodiments, immunostimulatory compositions of the present invention are administered in the form of a pharmaceutically acceptable salt. When used the salts should be pharmaceutically acceptable, but non-pharmaceutically

acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include, but are not limited to, acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives may include benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

In some embodiments, a composition comprising a NE and an immunogen is co-administered with one or more antibiotics. For example, one or more antibiotics may be administered with, before and/or after administration of a composition comprising a NE and an immunogen. The present invention is not limited by the type of antibiotic co-administered. Indeed, a variety of antibiotics may be co-administered including, but not limited to, β -lactam antibiotics, penicillins (such as natural penicillins, aminopenicillins, penicillinase-resistant penicillins, carboxy penicillins, ureido penicillins), cephalosporins (first generation, second generation, and third generation cephalosporins), and other β -lactams (such as imipenem, monobactams), β -lactamase inhibitors, vancomycin, aminoglycosides and spectinomycin, tetracyclines, chloramphenicol, erythromycin, lincomycin, clindamycin, rifampin, metronidazole, polymyxins, doxycycline, quinolones (e.g., ciprofloxacin), sulfonamides, trimethoprim, and quinolines.

There are an enormous amount of antimicrobial agents currently available for use in treating bacterial, fungal and viral infections. For a comprehensive treatise on the general classes of such drugs and their mechanisms of action, the skilled artisan is referred to Goodman & Gilman's "The Pharmacological Basis of Therapeutics" Eds. Hardman *et al.*, 9th Edition, Pub. McGraw Hill, chapters 43 through 50, 1996, (herein incorporated by reference in its entirety). Generally, these agents include agents that inhibit cell wall synthesis (e.g., penicillins, cephalosporins, cycloserine, vancomycin, bacitracin); and the imidazole antifungal agents (e.g., miconazole, ketoconazole and clotrimazole); agents that act directly to disrupt the cell membrane of the microorganism (e.g., detergents such as polmyxin and colistimethate and the antifungals nystatin and amphotericin B); agents that

affect the ribosomal subunits to inhibit protein synthesis (*e.g.*, chloramphenicol, the tetracyclines, erythromycin and clindamycin); agents that alter protein synthesis and lead to cell death (*e.g.*, aminoglycosides); agents that affect nucleic acid metabolism (*e.g.*, the rifamycins and the quinolones); the antimetabolites (*e.g.*, trimethoprim and sulfonamides); and the nucleic acid analogues such as zidovudine, gancyclovir, vidarabine, and acyclovir which act to inhibit viral enzymes essential for DNA synthesis. Various combinations of antimicrobials may be employed.

The present invention also includes methods involving co-administration of a composition comprising a NE and an immunogen with one or more additional active and/or immunostimulatory agents (*e.g.*, a composition comprising a NE and a different immunogen, an antibiotic, anti-oxidant, etc.). Indeed, it is a further aspect of this invention to provide methods for enhancing prior art immunostimulatory methods (*e.g.*, immunization methods) and/or pharmaceutical compositions by co-administering a composition of the present invention. In co-administration procedures, the agents may be administered concurrently or sequentially. In one embodiment, the compositions described herein are administered prior to the other active agent(s). The pharmaceutical formulations and modes of administration may be any of those described herein. In addition, the two or more co-administered agents may each be administered using different modes (*e.g.*, routes) or different formulations. The additional agents to be co-administered (*e.g.*, antibiotics, adjuvants, etc.) can be any of the well-known agents in the art, including, but not limited to, those that are currently in clinical use.

In some embodiments, a composition comprising a NE and immunogen is administered to a subject via more than one route. For example, a subject that would benefit from having a protective immune response (*e.g.*, immunity) towards a pathogenic microorganism may benefit from receiving mucosal administration (*e.g.*, nasal administration or other mucosal routes described herein) and, additionally, receiving one or more other routes of administration (*e.g.*, parenteral or pulmonary administration (*e.g.*, via a nebulizer, inhaler, or other methods described herein)). In some preferred embodiments, administration via mucosal route is sufficient to induce both mucosal as well as systemic immunity towards an immunogen or organism from which the immunogen is derived. In other embodiments, administration via multiple routes serves to provide both mucosal and systemic immunity. Thus, although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular

mechanism of action, in some embodiments, it is contemplated that a subject administered a composition of the present invention via multiple routes of administration (e.g., immunization (e.g., mucosal as well as airway or parenteral administration of a composition comprising a NE and immunogen of the present invention) may have a stronger immune response to an immunogen than a subject administered a composition via just one route.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compositions, increasing convenience to the subject and a physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer based systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Pat. No. 5,075,109, hereby incorporated by reference. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-di-and tri-glycerides; hydrogel release systems; sytastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which an agent of the invention is contained in a form within a matrix such as those described in U.S. Pat. Nos. 4,452,775, 4,675,189, and 5,736,152, each of which is hereby incorporated by reference and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Pat. Nos. 3,854,480, 5,133,974 and 5,407,686, each of which is hereby incorporated by reference. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

In preferred embodiments, a composition comprising a NE and an immunogen of the present invention comprises a suitable amount of the immunogen to induce an immune response in a subject when administered to the subject. In preferred embodiments, the immune response is sufficient to provide the subject protection (e.g., immune protection) against a subsequent exposure to the immunogen or the microorganism (e.g., bacteria or virus) from which the immunogen was derived. The present invention is not limited by the amount of immunogen used. In some preferred embodiments, the amount of immunogen (e.g., virus or bacteria neutralized by the NE, or, recombinant protein) in a composition comprising a NE and immunogen (e.g., for use as an immunization dose) is selected as that

amount which induces an immunoprotective response without significant, adverse side effects. The amount will vary depending upon which specific immunogen or combination thereof is/are employed, and can vary from subject to subject, depending on a number of factors including, but not limited to, the species, age and general condition (e.g., health) of the subject, and the mode of administration. Procedures for determining the appropriate amount of immunogen administered to a subject to elicit an immune response (e.g., a protective immune response (e.g., protective immunity)) in a subject are well known to those skilled in the art.

In some embodiments, it is expected that each dose (e.g., of a composition comprising a NE and an immunogen (e.g., administered to a subject to induce an immune response (e.g., a protective immune response (e.g., protective immunity)))) comprises 0.05-5000 μg of each immunogen (e.g., recombinant and/or purified protein), in some embodiments, each dose will comprise 1-500 μg , in some embodiments, each dose will comprise 350-750 μg , in some embodiments, each dose will comprise 50-200 μg , in some embodiments, each dose will comprise 25-75 μg of immunogen (e.g., recombinant and/or purified protein). In some embodiments, each dose comprises an amount of the immunogen sufficient to generate an immune response. An effective amount of the immunogen in a dose need not be quantified, as long as the amount of immunogen generates an immune response in a subject when administered to the subject. An optimal amount for a particular administration (e.g., to induce an immune response (e.g., a protective immune response (e.g., protective immunity))) can be ascertained by one of skill in the art using standard studies involving observation of antibody titers and other responses in subjects.

In some embodiments, it is expected that each dose (e.g., of a composition comprising a NE and an immunogen (e.g., administered to a subject to induce and immune response)) is from 0.001 to 15% or more (e.g., 0.001-10%, 0.5-5%, 1-3%, 2%, 6%, 10%, 15% or more) by weight immunogen (e.g., neutralized bacteria or virus, or recombinant and/or purified protein). In some embodiments, an initial or prime administration dose contains more immunogen than a subsequent boost dose

In some embodiments, when a NE of the present invention is utilized to inactivate a live microorganism (e.g., virus (e.g., HIV)), it is expected that each dose (e.g., administered to a subject to induce and immune response)) comprises between 10 and 10^9 pfu of the virus per dose; in some embodiments, each dose comprises between 10^5 and 10^8 pfu of the virus per dose; in some embodiments, each dose comprises between 10^3 and 10^5 pfu of the virus

per dose; in some embodiments, each dose comprises between 10^2 and 10^4 pfu of the virus per dose; in some embodiments, each dose comprises 10 pfu of the virus per dose; in some embodiments, each dose comprises 10^2 pfu of the virus per dose; and in some embodiments, each dose comprises 10^4 pfu of the virus per dose. In some embodiments, each dose comprises more than 10^9 pfu of the virus per dose. In some preferred embodiments, each dose comprises 10^3 pfu of the virus per dose.

In some embodiments, when a NE of the present invention is utilized to inactivate a live microorganism (e.g., a population of bacteria (e.g., of the genus *Bacillus* (*B. anthracis*))), it is expected that each dose (e.g., administered to a subject to induce and immune response)) comprises between 10 and 10^{10} bacteria per dose; in some embodiments, each dose comprises between 10^5 and 10^8 bacteria per dose; in some embodiments, each dose comprises between 10^3 and 10^5 bacteria per dose; in some embodiments, each dose comprises between 10^2 and 10^4 bacteria per dose; in some embodiments, each dose comprises 10 bacteria per dose; in some embodiments, each dose comprises 10^2 bacteria per dose; and in some embodiments, each dose comprises 10^4 bacteria per dose. In some embodiments, each dose comprises more than 10^{10} bacteria per dose. In some embodiments, each dose comprises 10^3 bacteria per dose.

The present invention is not limited by the amount of NE used to inactivate live microorganisms (e.g., a virus (e.g., one or more types of HIV)). In some embodiments, a 0.1% - 5% NE solution is used, in some embodiments, a 5%-20% NE solution is used, in some embodiments, a 20% NE solution is used, and in some embodiments, a NE solution greater than 20% is used order to inactivate a pathogenic microorganism. In preferred embodiments, a 10% NE solution is used.

Similarly, the present invention is not limited by the duration of time a live microorganism is incubated in a NE of the present invention in order to become inactivated. In some embodiments, the microorganism is incubated for 1-3 hours in NE. In some embodiments, the microorganism is incubated for 3-6 hours in NE. In some embodiments, the microorganism is incubated for more than 6 hours in NE. In preferred embodiments, the microorganism is incubated for 3 hours in NE (e.g., a 10% NE solution). In some embodiments, the incubation is carried out at 37°C . In some embodiments, the incubation is carried out at a temperature greater than or less than 37°C . The present invention is also not limited by the amount of microorganism used for inactivation. The amount of microorganism may depend upon a number of factors including, but not limited to, the total

amount of immunogenic composition (e.g., NE and immunogen) desired, the concentration of solution desired (e.g., prior to dilution for administration), the microorganism and the NE. In some preferred embodiments, the amount of microorganism used in an inactivation procedure is that amount that produces the desired amount of immunogen (e.g., as described herein) to be administered in a single dose (e.g., diluted from a concentrated stock) to a subject.

In some embodiments, a composition comprising a NE and an immunogen of the present invention is formulated in a concentrated dose that can be diluted prior to administration to a subject. For example, dilutions of a concentrated composition may be administered to a subject such that the subject receives any one or more of the specific dosages provided herein. In some embodiments, dilution of a concentrated composition may be made such that a subject is administered (e.g., in a single dose) a composition comprising 0.5-50% of the NE and immunogen present in the concentrated composition. In some preferred embodiments, a subject is administered in a single dose a composition comprising 1% of the NE and immunogen present in the concentrated composition. Concentrated compositions are contemplated to be useful in a setting in which large numbers of subjects may be administered a composition of the present invention (e.g., an immunization clinic, hospital, school, etc.). In some embodiments, a composition comprising a NE and an immunogen of the present invention (e.g., a concentrated composition) is stable at room temperature for more than 1 week, in some embodiments for more than 2 weeks, in some embodiments for more than 3 weeks, in some embodiments for more than 4 weeks, in some embodiments for more than 5 weeks, and in some embodiments for more than 6 weeks.

Generally, the emulsion compositions of the invention will comprise at least 0.001% to 100%, preferably 0.01 to 90%, of emulsion per ml of liquid composition. It is envisioned that the formulations may comprise about 0.001%, about 0.0025%, about 0.005%, about 0.0075%, about 0.01%, about 0.025%, about 0.05%, about 0.075%, about 0.1 %, about 0.25%, about 0.5%, about 1.0%, about 2.5%, about 5%, about 7.5%, about 10%, about 12.5%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95% or about 100% of emulsion per ml of liquid composition. It should be understood that a range between any two figures listed above is specifically contemplated to be encompassed within the metes and bounds of the present invention. Some variation in

dosage will necessarily occur depending on the condition of the specific pathogen and the subject being immunized.

In some embodiments, following an initial administration of a composition of the present invention (e.g., an initial vaccination), a subject may receive one or more boost administrations (e.g., around 2 weeks, around 3 weeks, around 4 weeks, around 5 weeks, around 6 weeks, around 7 weeks, around 8 weeks, around 10 weeks, around 3 months, around 4 months, around 6 months, around 9 months, around 1 year, around 2 years, around 3 years, around 5 years, around 10 years) subsequent to a first, second, third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, and/or more than tenth administration. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments, reintroduction of an immunogen in a boost dose enables vigorous systemic immunity in a subject. The boost can be with the same formulation given for the primary immune response, or can be with a different formulation that contains the immunogen. The dosage regimen will also, at least in part, be determined by the need of the subject and be dependent on the judgment of a practitioner.

Dosage units may be proportionately increased or decreased based on several factors including, but not limited to, the weight, age, and health status of the subject. In addition, dosage units may be increased or decreased for subsequent administrations (e.g., boost administrations).

A composition comprising an immunogen of the present invention finds use where the nature of the infectious and/or disease causing agent (e.g., for which protective immunity is sought to be elicited) is known, as well as where the nature of the infectious and/or disease causing agent is unknown (e.g., in emerging disease (e.g., of pandemic proportion (e.g., influenza or other outbreaks of disease))). For example, the present invention contemplates use of the compositions of the present invention in treatment of or prevention of (e.g., via immunization with an infectious and/or disease causing HIV or HIV-like agent neutralized via a NE of the present invention) infections associated with an emergent infectious and/or disease causing agent yet to be identified (e.g., isolated and/or cultured from a diseased person but without genetic, biochemical or other characterization of the infectious and/or disease causing agent).

It is contemplated that the compositions and methods of the present invention will find use in various settings, including research settings. For example, compositions and

methods of the present invention also find use in studies of the immune system (e.g., characterization of adaptive immune responses (e.g., protective immune responses (e.g., mucosal or systemic immunity))). Uses of the compositions and methods provided by the present invention encompass human and non-human subjects and samples from those subjects, and also encompass research applications using these subjects. Compositions and methods of the present invention are also useful in studying and optimizing nanoemulsions, immunogens, and other components and for screening for new components. Thus, it is not intended that the present invention be limited to any particular subject and/or application setting.

The formulations can be tested *in vivo* in a number of animal models developed for the study of mucosal and other routes of delivery. As is readily apparent, the compositions of the present invention are useful for preventing and/or treating a wide variety of diseases and infections caused by viruses, bacteria, parasites, and fungi, as well as for eliciting an immune response against a variety of antigens. Not only can the compositions be used prophylactically or therapeutically, as described above, the compositions can also be used in order to prepare antibodies, both polyclonal and monoclonal (e.g., for diagnostic purposes), as well as for immunopurification of an antigen of interest. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) can be immunized with the compositions of the present invention. The animal is usually boosted 2-6 weeks later with one or more--administrations of the antigen. Polyclonal antisera can then be obtained from the immunized animal and used according to known procedures (See, e.g., Jurgens et al., J. Chrom. 1985, 348:363-370).

In some embodiments, the present invention provides a kit comprising a composition comprising a NE and an immunogen. In some embodiments, the kit further provides a device for administering the composition. The present invention is not limited by the type of device included in the kit. In some embodiments, the device is configured for nasal application of the composition of the present invention (e.g., a nasal applicator (e.g., a syringe) or nasal inhaler or nasal mister). In some embodiments, a kit comprises a composition comprising a NE and an immunogen in a concentrated form (e.g., that can be diluted prior to administration to a subject).

In some embodiments, all kit components are present within a single container (e.g., vial or tube). In some embodiments, each kit component is located in a single container (e.g., vial or tube). In some embodiments, one or more kit component are located in a single

container (e.g., vial or tube) with other components of the same kit being located in a separate container (e.g., vial or tube). In some embodiments, a kit comprises a buffer. In some embodiments, the kit further comprises instructions for use.

EXAMPLES

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Example 1

Materials and methods: Nanoemulsion inactivated Vaccinia virus

Animals. Pathogen-free, 5 to 6-week-old, female Balb/c mice were purchased from Charles River Laboratories. Vaccination groups were housed separately, five animals to a cage, in accordance with the American Association for Accreditation of Laboratory Animal Care standards. All procedures involving mice were performed according to the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Viruses. Two exemplary vaccinia viruses (VV) were used during the development of the present invention, VV_{WR} and VV_{WR-Luc}. VV_{WR} (NIH TC-adapted) was obtained from the American Type Culture Collection (ATCC). Recombinant VV_{WR-Luc} expresses firefly luciferase from the p7.5 early/late promoter and has been described (See, e.g., Luker et al., *Virology*. 2005, 341(2):284-300). VV_{WR-Luc} is not attenuated *in vitro* or *in vivo* because the virus was constructed with a method that does not require deletion of any viral genes (See, e.g., Blasco and Moss (1995). *Gene* 158(2), 157-162; Luker et al., *Virology*. 2005, 341(2):284-300).

Stocks of all viruses were generated using the method of Lorenzo et al (See, e.g., Lorenzo et al., *Methods Mol Biol*. 2004; 269:15-30) with some modification. Virus was propagated on Vero cells infected at a multiplicity of infection of 0.5. Cells were harvested at 48 to 72 h and virus was isolated from culture supernatants and cells lysates. Cell lysates were obtained by rapidly freeze-thawing the cell pellet followed by homogenization in Dounce homogenizer in 1mM Tris pH 9.0. Cell debris was removed by centrifugation at 2000 rpm. The purified virus stocks were obtained from clarified supernatants by layering on 4% to 40% sucrose gradients which were centrifuged for 1 hr at 25,000 x g. Turbid bands, containing viral particles, were collected, diluted in 1mM Tris pH 9 and then concentrated by 1hr centrifugation at 25,000 x g. Viral pellets were re-suspended in 1mM

Tris pH 9 and stored frozen at -80°C as virus stock. The VV_{WR} stocks were titered on Vero cells (See, e.g., Myc et al., Vaccine. 21:3801-3814). VV_{WR} has identical surface proteins as the native strain, but expresses luciferase protein during infection. This allowed for a sensitive cytotoxicity and morbidity assessment and the monitoring of the viral infection in animals with imaging techniques. Comparison of the serological response in VV_{WR} immunized animals either by ELISA, Western blot or virus neutralization assays showed no difference in titer to either VV_{WR} or VV_{WR-Luc}.

Nanoemulsion (NE). NE (W₂₀5EC) was obtained from NANOBIO Corporation, Ann Arbor, MI (See U.S. Patent No. 6,015,832 issued to NANOBIO Corporation (Ann Arbor, MI), herein incorporated by reference in its entirety). The nanoemulsion was manufactured by emulsification of cetyl pyridium chloride (1%), Tween 20 (5%) and Ethanol (8%) in water with soybean oil (64%) using a high speed emulsifier. Resultant droplets have a mean particle size of 300 $\pm$ 25 nm in diameter. W₂₀5EC has been formulated with surfactants and food substances considered "Generally Recognized as Safe" (GRAS) by the FDA. W₂₀5EC can be economically manufactured under Good Manufacturing Practices (GMP) and is stable for at least 18 months at 40°C.

Preparation of the NE-based vaccine. Vaccinia virus (VV) neutralization data generated during the development of the present invention indicated that 1 hr incubation with 10% NE or 0.1% formalin was sufficient for inactivation of the virus (e.g., six log VV titer reduction). On the basis of these results several formulations (e.g., compositions) for inducing an immune response (e.g., vaccine formulations) were produced for animal immunization. The compositions (e.g., for stimulating an immune response) were prepared as follow: To assure complete virus neutralization (e.g., virus inactivation) for the NE-killed VV, samples containing 1×10^3 pfu to 5×10^5 pfu per dose of VV were incubated for 3 hrs at 37°C in 10% W₂₀5EC NE, and were subsequently diluted to 1% NE for intranasal instillation (e.g., 1×10^3 to 1×10^5 pfu per dose). For the vaccine formulations containing formalin-killed virus, the formalin (Sigma) inactivation of VV was performed at RT for 3 hrs in 0.1% formalin. Formalin-killed virus was diluted in either saline or 1% NE to 10^3 or 10^5 pfu per dose to reduce the formalin to nontoxic concentrations for intranasal immunization. For every formulation in each experiment, virus inactivation by either NE or fomalin was confirmed in vitro by infecting Vero cells, followed with two subsequent passages of culture supernatants after 3-4 days of incubation. None of the control infections showed a presence of viral plaques. Additionally, PCR-detection assays of viral DNA in

Vero cells and lungs of treated animals were performed as described below to confirm the absence of live, replicating virus.

Immunization. Samples of pre-immune serum were collected from mice prior to initial immunization. All animals were anesthetized with isoflurane and vaccinated (e.g., with 10-15 μ l of vaccine formulation per nare) using a pipette tip. Emulsion was administered slowly to minimize the swallowing of material. After vaccination, animals were observed for adverse reactions. Specific anti-VV antibody response was measured in blood samples 3 weeks after the initial (e.g., prime) administration (e.g., immunization) and at two to three week intervals after the second and third administrations (e.g., immunizations) when additional administrations were performed.

Immunization by scarification was performed in anesthetized mice by superficial scarification at the base of the tail. Before the procedure, hair was removed by a clipper to expose approximately 0.5-0.7 square centimeters and the naked skin was disinfected with 70% ethanol. A sterile bifurcate needle was used to superficially abrade the epidermis and 1×10^5 pfu dose of live VV_{WR} was applied in 10 μ l PBS. Animals were immobilized for up to 10 minutes to ensure virus absorption into the skin.

Bioluminescence imaging. Bioluminescence imaging was performed with a cryogenically-cooled CCD camera (IVIS) as described elsewhere (See, e.g., Luker et al., (2002). *J Virol* 76(23), 12149-12161; Cook and Griffin, (2003). *J Virol* 77(9), 5333-5338). Data for photon flux were quantified by region-of-interest (ROI) analysis of the head, chest and abdomen of infected mice. Background photon flux from an uninfected mouse injected with luciferin was subtracted from all measurements.

Collection of blood, bronchial alveolar lavage (BAL) and splenocytes. Blood samples were obtained from the saphenous vein at various time points during the course of trials conducted during the development of the present invention. Final samples were obtained by cardiac puncture from euthanized, premorbid mice. Serum was obtained from blood by centrifugation at 1500 x g for 5 minutes after the blood coagulated for 30-60 minutes at room temperature. Serum samples were stored at -20°C until used.

BAL fluid was obtained from mice euthanized by isoflurane inhalation. After the trachea was dissected, a 22 gauge catheter (Angiocath, B-D) attached to a 1 ml syringe was inserted into the trachea. The lungs were infused twice with 0.5 ml of PBS containing 10 μ M DTT and 0.5 mg/ml aprotinin. Approximately 1.0 ml of aspirate was recovered with a syringe. BAL samples were stored at -20°C until analyzed.

Murine splenocytes were mechanically isolated to obtain single-cell suspension in PBS. Red blood cells (RBC) were removed by lysis with ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA), and the remaining cells washed twice in PBS. For antigen-specific proliferation or cytokine expression assays, splenocytes (2-4 x 10⁶ /ml) were resuspended in RPMI 1640 medium supplemented with 5 % FBS, 200nM L-glutamine, and penicillin/streptomycin (100U/ml and 100µg/ml).

PCR detection of viral DNA. Forward primer (SEQ ID NO. 1: 5'-ATG ACA CGA TTG CCA ATA C 3') and reverse primer (SEQ ID NO. 2: 5'-CTA GAC TTT GTT TTC TG 3') were used (See, e.g., Ropp et al., J. Clin. Microbiol., 1995: 2069-2076). These primers are for conserved regions of the HA gene of all orthopox viruses (e.g., VV) and were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). DNA was isolated from Vero cells or from lung tissue homogenates with Triagent per the manufacturer's protocol (MRC, Cincinnati, OH). PCR amplification was performed with 10 µg of total cell or lung DNA using 0.5 µM of each primer, 0.2 mM of each dNTP, 2.5 mM of MgCl₂, and 0.1 U/µl of *Taq* DNA Polymerase (ROCHE Molecular Biochemicals, Indianapolis, IN). PCR reactions were carried out in a total volume of 20 µl, incubated at 94°C for 1 min, followed by 25 cycles with annealing at 55°C, extension at 72°C and denaturation at 94°C. PCR product analysis was performed using electrophoresis on 1% agarose gel in Tris-borate buffer for electrophoresis and ethidium bromide for DNA staining. Analysis was performed using a photoimaging camera and software from BioRad (Hercules, CA). Purified VV DNA (1 ng) mixed with lung DNA served as a positive control. Gel analysis was performed using a photoimaging camera and software from BIORAD (Hercules, CA).

Specific anti-virus IgA and IgG determination. Mouse anti-vaccinia antibodies were determined by ELISA. Microtiter 96-well flat bottom NUNC-PolySorp polystyrene plates were coated with a dilution of infected Vero cells lysate containing at least 5 × 10⁴ pfu/well of vaccinia virus in PBS. Plates were incubated overnight at 4°C and fixed with 50% mixture of ethanol/acetone (EtOH/acetone) for 1 hour at -20°C. After the fixing solution was removed, plates were washed twice with PBS containing 0.001% Tween 20 and then blocked for 1 hour at 37°C with 1% non-fat dry milk in PBS containing 0.2% Tween 20. Mouse sera or BAL fluid were serially diluted in PBS with 0.1% BSA, 100 µl aliquots were added to wells, and the plates were incubated for 2 hours at 37°C. Plates were washed three times with PBS-0.05% Tween 20, followed by 1 hour incubation with either anti-mouse IgG or anti-mouse IgA alkaline phosphatase (AP)-conjugated antibodies, and then washed three

times. The colorimetric reaction was performed with AP substrate SIGMAFAST (SIGMA, St. Louis, MO) according to the manufacturer's protocol. Spectrophotometric readouts were done using the SPECTRAMAX 340 ELISA reader (MOLECULAR DEVICES, Sunnyvale, CA) at 405 nm and reference wavelength of 690 nm. The endpoint titers and antibody concentrations were calculated as the serum dilution resulting in an absorbance greater than two standard deviations above the absorbance in control wells. The IgG antibody concentration was calculated according to the logarithmic transformation of the linear portion of the standard curve generated with the AP-conjugated anti-IgG antibody and multiplied by the serum dilution factor. The serum antibody concentrations are presented as a mean value $\pm$ standard error (sem). Serum from the naive mice was used as a control for non-specific absorbance.

Anti-VV IgG antibody activity targeted toward alcohol denatured versus formalin-alkylated viral epitopes was measured using ELISA, as described above with a few modifications. The 96 well plates were coated with 1×10^5 pfu/well of purified vaccinia virus and incubated overnight at 4°C. After virus was removed, wells were treated for 1 hour with either 50% EtOH/acetone at -20°C or with 1% formalin solution in PBS at 4°C. Plates were washed and blocked as described above. Pooled sera from mice immunized with various formulations of vaccine (VV/NE, VV/Fk/NE, VV/Fk) and sera from mice which survived sub-lethal infection with live vaccinia virus (VV live) were serially diluted in 0.1% BSA, and 100 μ L aliquots were added to EtOH/acetone and to the formalin-fixed wells. The assay was performed as describe above for the anti-vaccinia IgG determination. The optical density (OD) values at 405 nm were compared between EtOH/acetone and formalin-fixed viral antigens. The differences in the activity of anti-vaccinia antibodies were evaluated by the ratio of IgG titers on EtOH/acetone versus formalin at the same OD 405 nm value.

Neutralizing antibodies. Neutralizing antibodies were determined with both a standard plaque reduction assay (PRA) (See, e.g., Newman et al., J. Chem. Microbiol. 2003, 3154-3157) and the inhibition of luciferase activity using recombinant VV_{WR-Luc}. The PRA was conducted by mixing 10 μ L of heat-inactivated mouse serum in serial, two-fold dilutions with 10 μ L of serum-free RPMI medium containing 200-300 pfu of VV. Sera were incubated 6 hr at 37°C and subsequently placed in 0.5 ml of serum-free medium an overlaid on Vero cell monolayer. After 1 hr incubation, virus/serum inocula were removed and a fresh medium was placed on the cell monolayers. After 48 to 72 hrs, cells were fixed and stained

with 0.1% crystal violet. Plaques were counted by two independent observers and the neutralization titer calculated using non-immune serum as a control.

For the assessment of neutralization titer with VV_{WR-Luc}, 10 μ l of heat-inactivated mouse serum in serial, two-fold dilutions were mixed with 10 μ l of serum-free RPMI medium containing 2×10^3 pfu of virus. As in the PRA based neutralization assay, samples were incubated for 6 hr at 37°C, resuspended in 100 μ l of serum-free RPMI and incubated for 1 hr with Vero cells in 24 well plates. After 24-36 hrs, infected cells were lysed and virus-dependent luciferase activity was assessed by the luciferase assay described above. Neutralization titers (NT₅₀) were calculated from the luciferase inhibition curves using non-immune sera and virus in PBS as controls. Correlations between PRA and luciferase inhibition activity was made for each sample.

Vaccinia specific cytokine expression in splenocytes. Spleens from vaccinated mice were harvested 12 weeks after initial vaccination. Splenocytes were obtained from mechanically disrupted spleens and suspended at 3×10^6 cells/ml in RPMI 1640 supplemented with 5% FBS, L-glutamine and penicillin/streptomycin. Cells were incubated with either 1×10^3 or 1×10^4 pfu per well of vaccinia virus for 72 hours at 37°C. Cell culture supernatants were harvested and analyzed for cytokine production. PHA-P (1 μ g per well) was incubated with the cells as a positive control. The IFN- γ concentrations in splenocyte supernatants were determined using QUANTIKINE M ELISA kits (R&D SYSTEMS Inc., Minneapolis, MN) according to the manufacturer's directions.

Vaccinia virus challenge. Immunized mice were challenged with live vaccinia virus to evaluate the effectiveness of the vaccine. Serum samples were collected two days before the vaccinia challenge and animals were weighed on the day of the challenge. Aliquots of purified recombinant VV_{WR} or VV_{WR-Luc} (sonicated and titered before use) were thawed and diluted in saline the day of the challenge. Mice were anesthetized by inhalation of isoflurane and challenged intranasally with a 20 μ l suspension of 2×10^6 pfu live VV_{WR-Luc} corresponding to $10 \times \text{LD}_{50}$, or with live VV_{WR} doses ranging from 1×10^7 to 3.2×10^3 in 5 fold dilutions. Weight and body temperature were measured daily for 3 weeks following challenge. Mice that demonstrated a 30% loss in initial body weight were euthanized. Lethal dose (LD₅₀) and the infectious dose (ID₅₀) calculations were based on the animals death rates, and on the core body temperature and body weight loss, respectively (See, e.g., Reed and Muench, Am J Hyg 1938;27:493-7). Index of protection against lethal challenge (IP_{LD}) was calculated as follows: $\text{IP}_{\text{LD}} = \log_{10} \text{Maximum dose} - \log_{10} \text{LD}_{50} \text{ controls}$.

Similarly, index of protection against infection (IP_{ID}) was calculated as: $IP_{ID} = \log_{10}ID_{50}$ vaccinated - $\log_{10}LD_{50}$ controls.

Statistical analysis. Statistical analysis of the results was preformed using ANOVA, and Student's T-test for the determination of the *p value*.

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Example 2

Nasal immunization with nanoemulsion-inactivated Vaccinia virus results in the induction specific systemic IgG response

To evaluate virucidal activity of the NE *in vitro*, a range of NE concentrations was mixed with either VV_{WR} or VV_{WR-Luc} and incubated for 1 to 3 hours at 37°C. Results of both plaque reduction (PRA) and luciferase bioluminescence assays indicated NE concentration dependent inactivation of both viruses. The 10% NE completely inactivates greater than 10^6 pfu of vaccinia within 1 hour of incubation (See Figures 1A and B). Subsequent passages of the culture supernatants from cells infected with VV inactivated with 10% NE showed no evidence of surviving virus.

Complete inactivation of virus in the NE preparations was further demonstrated *in vivo* after intranasal administration of inactivated VV_{WR} using PCR amplification of DNA isolated from mice lungs after administration. No viral DNA was detected in any of the treated mice (See Figure 1C) while a control PCR (lung DNA spiked with VV_{WR} DNA) resulted in the product of the expected size > 950 bp. In addition, *in vivo* bioluminescence imaging of mice also indicated an absence of viral infection and no evidence of virus amplification after administration of 10^5 pfu of NE-inactivated VV_{WR-Luc}, as compared to a strong signal from mice nasally infected either 1×10^5 or 1×10^6 pfu of live VV_{WR-Luc} (See Figure 1D). Thus, *in vitro* and *in vivo* results indicated that incubation with 10% NE for at least 60 minutes causes complete inactivation of VV. Accordingly, all virus inactivations were performed with 10% NE for 3 hours and subsequently diluted to 1% NE for immunization.

Next, experiments were designed to evaluate if compositions of the present invention (e.g., NE-killed VV) could produce protective immunity similar to that seen in humans vaccinated by scarification with live, replicating VV (See, e.g., Hammarlund et al., Nat. Med. 2003, 9; 1131-1137). Mice were intranasally (i.n.) immunized with six formulations containing either 10^5 or 10^3 pfu doses of VV_{WR} killed with NE (10^5 /NE and 10^3 /NE, respectively), formalin-killed virus mixed with 1% NE (10^5 /Fk/NE and 10^3 /Fk/NE,

respectively), and formalin-killed virus in saline (10^5 /Fk and 10^3 /Fk). Control mice were treated with 1% NE alone. Antibody responses were characterized three weeks after initial vaccine administration (See Figure 2). Immunity was boosted with subsequent administrations, at 5 and 9 weeks (Figure 2A). Significant anti-VV IgG levels were detected after booster immunization in serum from mice vaccinated with either 10^5 /NE or 10^5 /Fk/NE with a mean anti-VV IgG concentrations of 1.5 μ g/ml and 1 μ g/ml, respectively. After a second boost, anti-VV antibody concentrations increased in all groups, and at the conclusion of the experiment (at 16 weeks), immunization with 10^3 /NE and 10^5 /NE, produced highest responses with mean concentrations of 44 μ g/ml and 70 μ g/ml of anti-VV IgG, respectively, followed by 10^5 /Fk/NE (17 μ g/ml). Immunizations with 10^3 /Fk/NE, and either 10^5 /Fk or 10^3 /Fk formulations of vaccine consistently produced low levels of anti-VV antibodies, which did not increase significantly after booster administrations (See Figure 2A). A comparison of a single-dose with a three-dose schedule of immunization with 10^5 /NE showed that a single dose of vaccine produced significant (~ 4 μ g/ml), albeit lower than three-dose, concentration of serum anti-VV IgG at 12 weeks after immunization. Thus, in some embodiments, the present invention provides that a single dose of VV/NE vaccine may be sufficient to initiate immune responses (e.g., mucosal or systemic immune responses), that can be enhanced by subsequent immunization (See Figure 2A insert). No specific anti-VV antibodies were detected in any of the control mice.

Analysis of cross-reactive anti-VV antibodies indicated that serum IgG from mice immunized with NE-killed virus reacted with both formalin-crosslinked (alkylated) and with alcohol-fixed (denatured but not alkylated) viral proteins. Vaccination with VV/NE vaccine produced anti-VV IgG with 25 fold higher reactivity to the native, non-alkylated epitopes (similar to serum from mice exposed to a live virus), and those antibodies were also effective in recognizing formalin fixed viral proteins. In contrast, sera from animals vaccinated with formalin-killed virus, either alone or mixed with NE, did not have increased reactivity with native VV epitopes.

Example 3

Subjects administered nanoemulsion-killed Vaccinia virus possess mucosal immunity to Vaccinia virus

Mucosal immunity was assayed by VV-specific secretory IgA antibody in bronchialalveolar fluids (BAL). Anti-VV IgA was detected in BAL from animals

immunized with either 10^3 /NE or 10^5 /NE. Animals vaccinated with formulations containing formalin-killed virus, whether diluted in saline or NE, did not produce measurable mucosal response despite the presence of serum anti-VV IgG (See Figure 2B). Thus, the present invention provides that a composition comprising NE-killed VV generates mucosal immunity in a subject (e.g., as demonstrated by the presence of VV-specific secretory IgA antibodies in the BAL of the subject) whereas compositions that do not contain NE-killed VV (e.g., formalin-killed VV) are not capable of generating mucosal immunity to VV.

Example 4

Serum and bronchial alveolar lavage (BAL) from subjects administered nanoemulsion-inactivated Vaccinia virus possess virus-neutralizing antibodies

The biological relevance of the anti-VV antibody response was assessed in the virus neutralization assays. Neutralizing activity was detected in the serum of some mice after the single vaccination (Figure 3A). However, consistent titers of serum neutralizing antibodies were present after two immunizations with either 10^5 /NE 10^3 /NE or 10^5 /Fk/NE. The mean 50% neutralization titer (NT_{50}) for each of these groups was ≥ 20 . In contrast, animals vaccinated with 10^3 /Fk/NE, 10^3 /Fk or 10^5 /Fk, virus neutralization was observed only in the lowest serum dilution. Subsequent immunization produced greater than a ten fold increase in NT_{50} titers, but only in the mice vaccinated with NE-killed virus (10^3 /NE and 10^5 /NE, $NT_{50} = 180$ and $NT_{50} = 500$, respectively). Third vaccination with any of formulations containing formalin-killed virus did not significantly increase VV neutralization. Significant neutralizing activity was also detected in BAL fluids from mice vaccinated with either 10^3 /NE or 10^5 /NE, and was lower in BAL from mice immunized with either 10^3 /Fk/NE or 10^5 /Fk/NE (See Figure 3 insert). Neutralizing activity was absent in BAL of mice immunized with formalin-killed virus diluted in saline and in the control, not vaccinated animals. Thus, the present invention provides that despite inactivation (e.g. complete neutralization) of VV, nanoemulsions comprising inactivated VV of the present invention retain important immunogenic epitopes (e.g., recognized and responded to by the immune system (e.g., humoral immune system) of a subject).

Example 5

Comparison of Response to Native VV-_{WR} and VV-_{WR-Luc}

VV-_{WR-Luc} has identical surface proteins as the native strain, but expresses luciferase protein during infection. This allows for mortality assessment and monitoring of viral infection in challenged animals with imaging techniques. Comparison of antibodies in VV-_{WR} immunized animals versus both viral strains either in ELISA, Western blot or virus neutralization assays showed no difference between VV-_{WR} and VV-_{WR-Luc}.

Example 6

Administration of NE-killed VV generates VV specific cellular immune responses

The effect of NE based vaccine on cellular response was explored using an *in vitro* cytokine expression assay in splenocytes. Individual cultures of mouse splenocytes were stimulated with 10^3 and 10^4 pfu of live vaccinia. VV-specific cellular immune responses were demonstrated by IFN- γ expression *in vitro* in splenocytes from animals immunized with either 10^3 /NE or 10^5 /NE. In contrast, no increase VV-specific IFN- γ production was observed in splenocytes from animals immunized with formalin-killed virus, even when was it was mixed with nanoemulsion. Production of IFN- γ in cells from mice treated with VV/NE vaccine indicates Th1 polarization of cellular response. No antigen specific cytokine expression was detected in control splenocyte cultures (See Figure 4).

Example 7

Subjects administered NE-killed VV are protected against challenge with live, infectious VV

Protective immunity produced by mucosal immunization was evaluated in the challenge studies. Three groups of mice were nasally immunized with three doses of either 10^5 /NE, 10^5 /Fk/NE or 10^5 /Fk vaccine. Control animals were treated with saline. At 12 weeks mice were challenged with $10 \times LD_{50}$ (2×10^6 pfu) of live VV_{WR-Luc}. Body weight and temperature were measured two times a day and animals were imaged for VV_{WR-Luc} luminescence once a day. All 10^5 /NE vaccinated mice survived viral challenge (See Figure 5A). Mice vaccinated with 10^5 /Fk/NE and 10^5 /Fk had 40% and 20% survival rates, respectively. Although not fully protective, vaccination with 10^5 /Fk/NE also extended mean time till death (TTD) from 5 to 7 days. None of the control mice survived challenge. Bioluminescence imaging used for assessment of viral infection demonstrated that two of the five 10^5 /NE immunized mice had minimally detectable virus replication which did not

affect their weight and body temperature while the other three had more progressive replication that resolved within 6 days after challenge (See Figure 5B). However, none of these animals had clinical evidence of infection. In contrast, all non-vaccinated controls became ill and died or were humanely euthanized within 4 to 7 days of virus challenge. These animals had massive virus replication and spreading of the infection throughout the nasopharyngeal passage, lung and abdomen as presented in photon flux data. In 10^5 /NE vaccinated mice, a low grade infection after i.n. challenge was limited to the head (nose) of vaccinated animals, without spreading to the chest and abdomen (See Table 4 below).

	Days	Vaccinated		Controls	
		avg	sem	avg	se
Head	2	7.0	3.3	33.8	12
	3	26.1	13.1	135.9	86
	4	75.2	16.5	431.0	25
	5	56.6	17.1	924.7	35
Chest	2	8.2	1.8	24.0	3
	3	13.3	2.0	119.8	25
	4	22.1	6.3	216.3	62
	5	16.6	1.3	618.2	13
Abdomen	2	12.1	1.0	19.2	5
	3	13.7	1.3	23.8	8
	4	23.0	2.8	28.3	10
	5	14.8	1.8	32.1	6

Table 4

Taken together, the imaging studies suggested an inverse correlation between the dissemination of infection and survival. The presence of self-limiting infection in some immunized mice correlated with the levels of neutralizing antibodies in the individual animals.

To further investigate effectiveness of mucosal NE-based vaccine, the i.n. immunization with three doses of 10^5 /NE was compared with vaccination by scarification with live VV_{WR} (10^5 /sc). At 12 weeks mice were i.n. challenged with the escalating doses

of live VV_{WR}. Survival data indicate that mucosal vaccination produced protective immunity equal to vaccination by scarification which is typically used for the human smallpox vaccine (See Table 5, below).

Challenge		Survival ^a		
Dose [pfu]	x LD ₅₀ ^b	NE vaccine	Scarification	Controls
1.0.E+07	77	5/5	5/5	0/5
2.0.E+06	15	5/5	5/5	0/5
4.0.E+05	3	5/5	5/5	1/5
8.0.E+04	0.62	5/5	5/5	3/5
1.6.E+04	0.12	5/5	5/5	5/5
3.2.E+03	0.02	5/5	5/5	5/5

^a - presented as a ratio of surviving to all mice

^b - calculated 1 x LD₅₀ was 5.13 x 10⁵ pfu of VV_{WR}.

Table 5

All mice vaccinated either with mucosal VV/NE vaccine or by scarification survived intranasal challenges with the maximal dose of 1 × 10⁷ pfu of VV_{WR} (77 × LD₅₀). Index of protection against lethal challenge (IP_{LD}) was 1.9 for both the NE-based vaccine and scarification. All control non-vaccinated animals died after challenge with 15 × LD₅₀ VV_{WR}. The high level of protection attained with i.n immunization was also seen in weight loss analysis of surviving mice. Although mucosal vaccination did not completely protect mice against respiratory infection with high doses of VV_{WR} (See Table 6, below), animals immunized with NE vaccine did not have clinical evidence of illness and had average weight loss of 10 % or less, whereas surviving mice in control groups lost more than 25% of weight at much lower doses of VV_{WR}. Statistical analysis indicated differences with *p* value <0.01 between body weight of immunized and control mice. Index of protection against infection (IP_{ID}) was 1 for VV/NE vaccine and 2.2 for scarification.

Challenge		Protected mice ^a		
Dose [pfu]	x LD ₅₀ ^b	NE vaccine	Scarification	Controls
1.0.E+07	77	0/5	3/5	0/5
2.0.E+06	15	0/5	5/5	0/5
4.0.E+05	3	1/5	5/5	0/5
8.0.E+04	0.62	2/5	5/5	0/5
1.6.E+04	0.12	4/5	5/5	0/5
3.2.E+03	0.02	5/5	5/5	5/5

a - presented as a ratio of mice which did not have decrease in body weight and temperature at any time after challenge to all mice

b - calculated 1 x LD₅₀ was 5.13 x 10⁵ pfu of VV_{WR}.

Table 6

Example 8

Materials and methods: rPA/NE Vaccine

Animals. Pathogen-free, female Balb/c, CBA/J mice (5-6 weeks old) and Hartley guinea pigs (females, 250 g) were purchased from Charles River Laboratories (Wilmington, MA). The mice and guinea pigs were housed in accordance with the American Association for Accreditation of Laboratory Animal Care standards. All procedures involving animals were performed according to the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan, the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch at Galveston, TX, and standard operating procedures at Battelle Memorial Institute, Columbus, OH.

Reagents. Recombinant *B. anthracis* protective antigen (rPA) and lethal factor (rLF) were obtained from List Biological Laboratories, Inc. (Campbell, CA) and BEI Resource Repository (ATCC) as lyophilized preparations of purified proteins. After reconstitution in sterile MILLI-Q water (5 mg/ml), the aliquots were stored at -80°C. A 20-mer oligonucleotide (ODN) 5'-TCC ATG ACG TTC CGT ACG TT -3' (SEQ ID NO.:3) (See, e.g., Moldoveanu et al., 1999. 15:1469-1476), containing non-methylated CpG repeats, was synthesized by INTEGRATED DNA TECHNOLOGIES (IDT, Coralville, IA). The *E. coli* monophosphoryl lipid A (MPL A, #L-6638), PHA-P, BSA, DTT, and other chemicals used in buffers were purchased from SIGMA-ALDRICH Corporation (St. Louis, MO). The phosphate buffered saline (PBS), cell culture media, and fetal bovine serum (FBS) were

purchased from GIBCO (Grand Island, NY) and HYCLONE (Logan, UT), respectively. The bovine serum albumin (BSA), alkaline phosphatase (AP)-conjugated antibodies, goat anti-mouse IgG (#A-3562), and goat anti-mouse IgA (α chain specific, #A-4937) were purchased from SIGMA, and goat anti-mouse IgE HPR-conjugate was bought from BETHYL (#A90-115P, Montgomery, TX). The Cell Proliferation Kit (XTT) was purchased from ROCHE DIAGNOSTICS (New Jersey, NY).

rPA/Adjuvant Formulations. Nanoemulsion (formulation W₂₀5EC) was obtained from NANOBIO Corporation, Ann Arbor, MI. This nanoemulsion is manufactured by the emulsification of cetyl pyridium chloride (CPC, 1%), Tween 20 (5%), and ethanol (8%) in water with hot-pressed soybean oil (64%), using a high-speed emulsifier (e.g., prepared by a two-step procedure according to U.S. Patent No. 6,015,832 issued to NANOBIO Corporation (Ann Arbor, MI), herein incorporated by reference in its entirety). Other than the CPC, W₂₀5EC is formulated with surfactants and food substances considered 'Generally Recognized as Safe' (GRAS) by the FDA. W₂₀5EC can be manufactured under Good Manufacturing Practices (GMP) and is stable for at least 18 months at 40°C without any special storage conditions. Nanoemulsion diameter was determined by dynamic light scattering (DLS) using the NICOMP 380 ZLS (PSS NICOMP Particle Sizing Systems, Santa Barbara, CA). The mean droplet size was consistently below 400 nm.

rPA/nanoemulsion formulations were prepared 30 to 60 minutes prior to immunization by mixing rPA protein solution with NE, using saline as diluent. Mice immunization studies were performed using a 20 μ g dose of rPA mixed with nanoemulsion concentrations of 0.1%, 0.5%, 1% and 2%. For immunization with immunostimulants, 20 μ g rPA was mixed with either 5 μ g of MPL A or 10 μ g CpG oligonucleotides in saline. The rPA aluminum hydroxide formulation (Alu, # A-8222, SIGMA) was prepared following an adsorption procedure as described (See Little et al., 2004 Vaccine 22:2843-2852). Guinea pig immunization studies were performed with 10 μ g, 50 μ g and 100 μ g doses of rPA mixed with 1% NE and saline as diluent. The immunization volume was 10 μ l/nare for mice and 50 μ l/nare for guinea pigs.

PAGE Analysis of the Recombinant PA. 0.5 μ g of rPA protein in saline or mixed with 1% NE was analyzed on 10% NU PAGE NOVEX Bis-Tris gel (INVITROGEN, # NP0301BOX) using a X CELL SURELOCK Mini-Cell platform for electrophoresis. Silver staining was performed according to the INVITROGEN SILVERXPRESS (# LC6100) method. The size of rPA protein was determined with a molecular weight marker MARK12

(INVITROGEN; # LC5677).

In vitro Uptake of Antigen. Fluorescently labeled rPA protein was prepared using the FLUOROTAG FITC Conjugation Kit (#FITC1, SIGMA) according to manufacturer's protocol. Murine dendritic cells (Jaws II) were incubated for 30 min at 37°C with PA-FITC conjugate in PBS or with PA-FITC mixed in nanoemulsion. The 0.001% NE concentration was chosen to ensure full viability of cells growing as a monolayer culture. After incubation, cells were washed 3 times with PBS and fixed with 1.25% formalin in PBS. Cellular uptake was then analyzed by fluorescent microscopy. The microphotographs were taken with an OLYMPUS IX70 microscope with an IXFLA inverted reflected fluorescence observation attachment. The images were processed using the SPOT basic and SPOT advanced programs.

Immunization Procedures. For each experiment, groups of mice were immunized intranasally (i.n.) with either one or two administrations of experimental vaccine 3 weeks apart. Animals were monitored for adverse reactions, and antibody responses were measured at 3 to 4 week intervals over a period of up to 12 weeks. The immunizations were conducted by first anesthetizing the mice with Isoflurane, then holding them in an inverted position. rPA/NE mixes were applied to the nares with a pipette tip (10 µl per nare) and the animals were then allowed to inhale the material. Hartley guinea pigs were vaccinated intranasally (i.n.) with one or two administrations of vaccine (50 µl per nare) 4 weeks apart and antibody responses were measured at 3 to 4 week intervals over a period of up to 22 weeks.

Blood Collection, Bronchial Alveolar Lavage (BAL) and Splenocytes. Blood samples were obtained from the saphenous vein at various time points during the course of the trials. The terminal sample was obtained by cardiac puncture from euthanized, premorbid mice. Serum was obtained from blood by centrifugation at $1500 \times g$ for 5 minutes after allowing it to coagulate for 30 to 60 minutes at room temperature. Serum samples were stored at -20°C until analyzed.

BAL fluid was obtained from mice euthanized by Isoflurane inhalation. After the trachea was dissected, a 22GA catheter (ANGIOCATH, B-D) attached to a syringe was inserted into the trachea. The lungs were infused twice with 0.5 ml of PBS containing 10 µM DTT and 0.5 mg/ml aprotinin (protease inhibitors) and approximately 1 ml of aspirate was recovered. BAL samples were stored at -20°C for further study.

Murine splenocytes were mechanically isolated to obtain single-cell suspension in

PBS. Red blood cells (RBC) were removed by lysis with ACK buffer (150 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA), and the remaining cells were washed twice in PBS. For the antigen specific proliferation or cytokine expression assays, splenocytes ($2-4 \times 10^6/\text{ml}$) were resuspended in RPMI 1640 medium supplemented with 2% FBS, 200 nM L-glutamine, and penicillin/streptomycin (100 U/ml and 100 $\mu\text{g}/\text{ml}$).

Determination of Anti-PA IgG and IgA. Mouse anti-PA-specific IgG and IgA levels were determined by ELISA. Microtiter plates (NUNC) were coated with 5 $\mu\text{g}/\text{ml}$ (100 μl) of rPA in a coating buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4°C. After the protein solution was removed, plates were blocked for 30 minutes with PBS containing 1% dry milk. The blocking solution was aspirated and plates were used immediately or stored sealed at 4°C until needed. Serum and BAL samples were serially diluted in 0.1% BSA in PBS, and 100 $\mu\text{l}/\text{well}$ aliquots were incubated in rPA-coated plates for 1 hour at 37°C. Plates were washed three times with PBS-0.05% Tween 20, followed by 1 hour incubation with either anti-mouse IgG or anti-mouse IgA alkaline phosphatase (AP)-conjugated antibodies (ROCKLAND), then washed three times and incubated with AP substrate SIGMAFAST (SIGMA). The colorimetric reaction was stopped with 1 N NaOH according to the manufacturer's protocol, and readouts were performed using a SPECTRAMAX 340 ELISA reader (MOLECULAR DEVICES, Sunnyvale, CA) at 405 nm and the reference wavelength of 690 nm. The endpoint titers were recorded and, in case of BAL fluid, the final antibody concentrations were calculated (See Rhie et al., 2003 PNAS 100:10925-10930) from the standard curves obtained for each assay plate, using goat F(ab')₂ anti-mouse IgG as a capturing agent and known concentrations of mouse IgG and IgA immunoglobulins, and detected with anti-IgG or anti-IgA-AP conjugates. Guinea pig anti-PA IgG was determined by the same method, except that rabbit anti-guinea pig IgG alkaline phosphatase (AP)-conjugate was used for detection (ROCKLAND). Antibody concentrations are presented as the mean $\pm$ sem (standard error of the mean) of endpoint titers.

Dot-blot Detection of IgE. Saline rPA solution (2 μl , 5 $\mu\text{g}/\text{ml}$) was adsorbed onto NYTRAN membrane (0.2 μm pore, Schleicher and Schuell, Keene, NH) and air-dried 30 minutes at room temperature. The membrane was blocked with PBS with 1% dry milk for 30 minutes, then washed 3 times with PBS and air-dried. For IgE detection, pooled sera from all groups of animals were diluted 1:10, 1:20, 1:40 and 1:80 in PBS with 0.1% BSA. The duplicate samples (2 μl) of each dilution were placed over the antigen spots and

incubated at RT for 30 minutes. Following 3 washes in PBS, the dot-blot was incubated with a 1:1000 dilution of anti-mouse IgE horseradish peroxidase (HRP)-conjugated antibody. After 5 washes with PBS, the dot-blot was incubated with HRP substrate until dots were visible.

Lethal Toxin (LeTx) Cytotoxicity and Neutralizing Antibodies Assay. Neutralizing antibody assay was performed using serial dilutions of the sera incubated for 1 hour with the LeTx (0.1 µg/ml rPA and 0.1 µg/ml rLF in PBS). The antibody-toxin mixtures were then added to RAW264.7 (20,000-30,000 cells/well) and incubated for 4-6 hours at 37°C. Cell viability was assessed with XTT assay. The serum titers resulting in 50% protection against LeTx cytotoxicity (neutralizing concentration NC_{50}) were calculated from the cell viability curves and presented as the mean value of the individual sera.

Live Spore Challenge. Challenge experiments were performed at the BSL4 and BSL3 facilities at Battelle Memorial Institute (Columbus, OH) and at the University of Texas Medical Branch (Galveston, TX), respectively. The intradermal (i.d.) challenges were performed according to Battelle Study Number: 556-G607602. Briefly, *B. anthracis* (Ames strain) spores were enumerated and diluted for an i.d. spore challenge. A concentrated stock solution of Ames Battelle Lot B22 was diluted in sterile water to an anticipated concentration of 5×10^3 colony-forming units per ml (cfu/ml). On Study Day 0, guinea pigs were i.d. challenged with a target dose of ~500 spores (0.1 ml). Post-challenge enumeration of spores revealed the actual number to be 1380, which responds to i.d. 1000 LD_{50} . The guinea pigs were observed twice daily for 14 days following the challenge for signs of clinical disease or death. Deaths were recorded to the nearest observation period. All animals surviving the challenge were anesthetized for terminal blood collection and then euthanized on day 14 post-challenge. Intranasal (i.n.) challenges were performed according to the JWP-004-0012 Nasal Challenge SOP protocol. Briefly, *B. anthracis* (Ames strain) spores were enumerated and diluted in PBS without calcium in magnesium for an i.n. spore challenge. Anesthetized guinea pigs were challenged by intranasal administration of either 1.2×10^6 or 1.2×10^7 spores, which corresponds, respectively, to an intranasal $10 \times LD_{50}$ and $100 \times LD_{50}$ dose. Post-challenge observation of guinea pigs was performed as described above for intradermal challenge.

Proliferation Assay. The proliferation of mouse splenocytes was measured by an assay of the 5-bromo-2-deoxyuridine (BrdU) incorporation, using CELL PROLIFERATION ELISA, (ROCHE Molecular Biochemicals, Mannheim, Germany). The cells were

incubated in the presence of rPA (5 µg/ml) or PHA-P mitogen (2 µg/ml) for 48 hours and then pulsed with BrdU for 24 hours. Cell proliferation was measured according to the manufacturer's instructions using a SPECTRA MAX 340 ELISA Reader at 370 nm and a reference wavelength of 492 nm.

Analysis of in vitro Cytokine Expression. Freshly isolated mouse splenocytes were seeded at 2×10^6 cells/0.5 ml (RPMI 1640, 2% FBS) and incubated with rPA (5 µg/ml) or PHA-P mitogen (2 µg/ml) for 72 hours. Cell culture supernatants were harvested and analyzed for the presence of cytokines. The IL-2, IL-4, IFN-γ and TNF-α cytokine assays were performed using QUANTIKINE ELISA kits (R&D SYSTEMS, Inc., Minneapolis, MN), according to the manufacturer's instructions.

Statistical Analysis. Data from individual experiments were expressed as mean ± standard error of the mean. Statistical significance was determined by ANOVA analysis of variance using the Student t and Fisher exact tests. All tests were at 95% confidence (two-tailed). A p value <0.05 was considered to be statistically significant.

Example 9

Physical Properties of Mixed rPA/NE Vaccine.

Mixing rPA with nanoemulsion (NE) did not appear to alter the antigen's protein structure, as it remained a single discrete band on a non-denaturing PAGE, corresponding to intact, full-length protein with a molecular weight of 83 kD (See Figure 6A). NE appeared to improve the stability of the rPA, which prevented the progressive degradation due to deamidation that is observed of the antigens incubated in a buffer solution (See, e.g., Gupta et al., 2003. FEBS Letters 554:505-510; Zomber et al., 2005 Journal of Biological Chemistry 280:39897-39906). The addition of the rPA protein did not alter either the size (359 +/-109 nm), appearance, or stability of the nanoemulsion as shown in photomicrographs of NE alone and mixed with antigen (See Figure 6B).

Example 10

Nanoemulsion Increases the Uptake of rPA by Dendritic Cells Without Inducing an Inflammatory Response.

Histopathology of nasal mucosa of mice intranasally immunized with either NE alone or mixed with rPA showed no evidence of an inflammatory response at 24 hour

intervals up to 4 days post administration. In contrast, *in vitro* studies documented that mixing rPA with NE significantly increased the uptake of protein in Jaws II dendritic cells (See Figure 7). Accordingly, in some embodiments, the present invention provides that NE adjuvant activity *in vivo* involves an increase in the antigen uptake by antigen presenting cells to nasal mucosa (e.g., without the indications of inflammation).

Example 11

rPA/NE Immunization Induces Serum Anti-PA Antibodies.

The effect of the nanoemulsion adjuvant on antibody response was measured in CBA/J and Balb/c mice. CBA/J mice were immunized intranasally with 20 μ g rPA mixed with either 0.1%, 0.5%, 1%, or 2% concentrations of NE. A rapid induction of anti-PA antibodies in serum was obtained in all vaccinated animals with some dependence on the concentration of the nanoemulsion. All CBA/J mice developed high titers of serum anti-PA IgG (endpoint titers ranging 10^4 to 10^5) at 5 weeks after only two administrations of the vaccine (at day 1 and at 3 weeks). Further assays at 8-12 weeks indicated that while there were lower titers in animals immunized with the 0.1% and 0.5% NE, there was no statistical difference between titers in animals immunized with either 1% or 2% rPA/NE. In contrast, no seropositive mice were found in animals intranasally immunized with rPA in saline (See Figure 8A).

The pattern of the IgG subtype antibodies indicated a prevalence of IgG2a and IgG2b over IgG1. Accordingly, in some embodiments, administration rPA mixed with NE to a subject induces Th1 polarization of the immune response (See Figure 8A, insert). To further characterize the immune response generated by intranasal nanoemulsion, Balb/c mice were immunized with 20 μ g rPA mixed with 1% NE (rPA/NE) and compared to immunization with 20 μ g rPA mixed with either MPL A (rPA/MPL A), unmethylated CpG ODN (rPA/CpG) or aluminum hydroxide (rPA/Alu) (See, e.g., Peterson et al., 2006. *Infection and Immunity* 74:1016-1024; Pittman et al., 2001. *Vaccine* 20:972-978; Pittman et al., 2002. *Vaccine* 20:2107-2115; Reuveny et al., 2001. *Infect Immun* 69:2888-2893). After two administrations of each formulation all mice immunized with rPA/NE were seropositive, with anti-PA IgG endpoint titers of at least 10^5 . This was compared to titers ranging from 10^2 - 10^3 in the rPA/MPL A, rPA/CpG and rPA/Alu immunization groups (See Figure 8B). No anti-PA antibodies were detected in animals nasally immunized with rPA in saline.

Serum was also analyzed for the presence of anti-PA IgE antibodies and revealed IgE anti-PA (detectable in at least 1:80 dilution in dot blots) in mice intramuscularly immunized with rPA/Alu, but not in any other group (See Figure 8B, insert). This is consistent with reports of alum adjuvant-based vaccines inducing a Th2 response (See, e.g., Johansson et al., 2004. Vaccine 22:2873-2880; Lindblad, 2004. Vaccine 22:3658-3668).

Example 12

Intranasal rPA/NE Vaccination Produces Mucosal Immunity.

It was determined whether nasal immunization could induce mucosal immunity (e.g., that protects against respiratory infection (See, e.g., Davis, 2001 Advanced Drug Delivery Reviews 51:21-42; Zuercher, 2003. Viral Immunology 16:279-289). Significant levels of anti-PA-specific secretory IgA antibodies were observed in bronchial lavage (BAL) samples from Balb/c mice vaccinated with rPA/NE (See Figure 9A). A similar pattern, with higher antibody concentrations, was detected for anti-PA IgG in BAL (See Figure 9B). The animals with titers of secretory IgA in BAL also had detectable levels of serum anti-PA IgA. Thus, the present invention provides that significant mucosal immune responses are induced via nasal administration of a vaccine comprising rPA in NE, but not with intramuscular immunization. No inflammatory response was observed in histopathological examination of animals' nasal mucosa after administration of NE with or without antigen, indicating that the nanoemulsion is not pro-inflammatory.

Example 13

rPA/NE Vaccines Produce Neutralizing Antibodies Against Anthrax Toxin in Mice.

In order to evaluate whether mucosal nanoemulsion-based vaccine could produce toxin neutralizing antibodies, sera from immunized mice were tested for the ability to neutralize anthrax lethal toxin (LeTx). Sera from mice immunized with rPA/NE were effective in neutralizing LeTx and prevented RAW264.7 cell death with an $NC_{50} > 10^3$. In contrast, sera from mice immunized with either rPA/MPL A, rPA/CpG or rPA/Alu had $NC_{50} < 10$. Naive control sera or sera from mice immunized with rPA in saline did not inhibit LeTx cytotoxicity at any concentration (See Figure 10).

Example 14

rPA/NE Immunization Yields Th1 Cellular Responses.

PA antigen-specific cellular responses were measured in a proliferation assay (See Figure 5) and through the analysis of cytokine secretion from splenocytes stimulated *in vitro* with rPA (See Table 7 below). As shown in Figure 11, rPA stimulated proliferation in splenocytes obtained from mice immunized with rPA/NE. No antigen-specific proliferation was detected in splenocytes from animals immunized with either rPA alone or rPA with CpG ODNs.

PA-activated spleen cells showed extensive production of INF- γ , TNF- α , and IL-2, but failed to produce IL-4 when compared to control (non-stimulated) cells. Thus, in some embodiments, the present invention provides that immunization (e.g., nasal administration) with rPA/NE yields specific Th1-type polarized cellular responses (See Table 7). In contrast, splenocyte cultures incubated with PHA induced significant proliferation and the secretion of both Th1 and Th2 cytokines.

	PA/NE		Ctrl	
	- PA	+ PA	- PA	+PA
IFN- γ	26.37 $\pm$ 8.10	93.30 $\pm$ 24.40*	13.23 $\pm$ 2.33	15.40 $\pm$ 1.1
TNF- α	4.74 $\pm$ 2.62	22.62 $\pm$ 12.85*	3.51 $\pm$ 2.34	2.50 $\pm$ 2.1
IL-2	27.10 $\pm$ 2.62	59.00 $\pm$ 16.9*	33.00 $\pm$ 17.30	35.00 $\pm$ 1.1
IL-4	17.44 $\pm$ 2.17	21.76 $\pm$ 9.76	19.00 $\pm$ 2.73	18.17 $\pm$ 1.1

Table 7

Example 15

rPA/NE Vaccines Protect Guinea Pigs Against Intradermal Live Spore Challenge.

Three groups of guinea pigs were vaccinated intranasally with 10, 50, and 100 μ g doses of rPA mixed with 1% NE. IgG responses were observed after a single vaccination and continued to increase after a second administration (at 4 weeks), producing endpoint antibody titers $>1 \times 10^5$. The animals were subsequently followed for 6 months to evaluate the duration of immunity. Nasal immunization in these animals produced durable immune responses with high antibody titers ($>10^4$) for at least 6 months (See Figure 12A). At 6 months, the animals were challenged intradermally (i.d.) with $1000 \times \text{LD}_{50}$ Ames strain spores. Survival data indicate that mucosal vaccination of guinea pigs with any of the three

concentrations of rPA in NE produced 100% protection against the i.d. challenge, while none of the control animals survived (See Figure 12B). A LeTx neutralization assay before the challenge documented mean serum NC₅₀ titers of 3×10^2 in the 10 µg rPA/NE group and NC₅₀ ~ 1×10^3 in both the 50 µg and the 100 µg rPA/NE immunized groups (See Figure 12B, insert).

Example 16

rPA/NE Vaccines Protect Against Intranasal Spore Challenge.

The protective effect of intranasal immunization was also tested in an inhalation challenge trial. Three groups of guinea pigs were immunized with formulations containing 10, 50, and 100 µg rPA mixed with 1% NE. Immunization produced 100% seroconversion and significant anti-PA IgG responses in immunized animals. A boost at 4 weeks resulted in the rapid increase of anti-PA IgG in the serum, producing endpoint antibody titers $>1 \times 10^5$ in all groups. A LeTx neutralization assay before the challenge indicated a mean NC₅₀ titers of $1-2 \times 10^3$ in all vaccinated groups (See Figure 13A). At 7 weeks the animals were challenged intranasally with either $10 \times \text{LD}_{50}$ (1.2×10^6 spores) or $100 \times \text{LD}_{50}$ (1.2×10^7 spores) of *B. anthracis* Ames strain spores. While none of the control guinea pigs survived, intranasal immunization with each formulation of rPA/NE produced protective immunity. rPA/NE immunizations yielded survival rates of 70% after the $10 \times \text{LD}_{50}$ challenge and 40% after the $100 \times \text{LD}_{50}$ challenge (See Figure 13B and 13C, respectively). Results of the $10 \times \text{LD}_{50}$ challenge provide that mucosal rPA/NE vaccine produced protective immunity comparable to i.m. vaccination using rPA with alum (See, e.g., Patton et al., 2006. ASM Bodefense Meeting: Abstract 232). Although there was no difference in the overall survival of the guinea pigs vaccinated with rPA/NE in a range of rPA concentrations, there was a significant, dose-dependent extension of the mean time until death (TTD) in the immunized animals (See Table 8, below).

Vaccination	Challenge 10 x LD ₅₀		Challenge 100 x LD ₅₀	
	Mortality	TTD	Mortality	TTD
PBS Ctrl	10/10	3.8 ± 1.4	10/10	3.1 ± 1.1
NE Ctrl	nd	nd	10/10	3.5 ± 1.1
10 µg/NE	3/10	7.0 ± 1.4*	6/10	4.3 ± 1.2
50 µg/NE	3/10	8.3 ± 1.2*	6/10	8.3 ± 1.2*
100 µg/NE	3/10	9.7 ± 3.1*	6/10	6.0 ± 1.4*

Table 5

Example 17**Material and Methods**

Animals. Pathogen-free, female Balb/c mice (5-6 weeks old) and Hartley guinea pigs (females, 250 g) were purchased from Charles River Laboratories (Wilmington, MA). The mice (five to a cage) and guinea pigs (one per cage) were housed in accordance with the American Association for Accreditation of Laboratory Animal Care standards. All procedures involving animals were performed according to the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Reagents. Recombinant HIV gp120_{BaL} and gp120_{SF162} serotype proteins produced in yeast were obtained from Dr. Joseph Sodorski via Dr. David Markovitz (Harvard Medical School and University of Michigan, respectively). The 5 mg/ml aliquots of the protein solutions in a sterile saline were stored at -80°C until used. The synthetic V3 loop peptide (BaL) was obtained from Dr. Steven King (University of Michigan). The 20-mer oligonucleotide (ODN) 5'-TCC ATG ACG TTC CGT ACG TT -3' (SEQ ID NO.: 4) (See, e.g., Moldoveanu et al., Vaccine 1998;16(11-12):1216-24), containing non-methylated CpG repeats, was synthesized by INTEGRATED DNA TECHNOLOGIES (IDT, Coralville, IA). The *S. minnesota* monophosphoryl lipid A (MPL A, #L-6638), PHA-P, BSA, DTT, and other chemicals used in buffers were purchased from SIGMA-ALDRICH Corporation (St. Louis, MO). The saline solution, phosphate buffered saline (PBS), cell culture media, and fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY) and HYCLONE (Logan, UT), respectively. The alkaline phosphatase (AP)-conjugated antibodies, goat anti-mouse IgG (#A-3562), goat anti-mouse IgA (α chain specific, #A-4937) were purchased from SIGMA, and rabbit anti-guinea pig IgG was bought from ROCKLAND (#606-408).

Preparation of the gp120/adjuvant formulations. The oil-in-water nanoemulsion

(NE) used in these studies obtained from NANOBIO Corporation, Ann Arbor, MI. NE was produced by the emulsification of cetyl pyridium chloride (CPC, 1%), nonionic surfactant (5%), and ethanol (8%) in water with hot-pressed soybean oil (64%), using a high-speed emulsifier and prepared by a two-step procedure (See U.S. Pat. No. 6,015,832 to NANOBIO Corporation, Ann Arbor, MI, hereby incorporated by reference in its entirety for all purposes). Except for the CPC, this nanoemulsion is formulated with surfactant and food substances considered 'Generally Recognized as Safe' (GRAS) by the FDA. NE mean droplet size (about 300 +/- 25 nm) was determined by dynamic light scattering (DLS) using the NICOMP 380 ZLS (PSS NICOMP Particle Sizing Systems, Santa Barbara, CA)

gp120/NE formulations were prepared by mixing gp120 protein solution with NE, using saline as diluent. Mice immunization studies were performed with a 20 µg dose of gp120 mixed with 0.1%, 0.5% and 1% NE concentrations. For immunization with immunostimulants, either 5 µg of MPL A or 10 µg CpG ODN was added to the 20 µg gp120 in 1% NE or to the 20 µg gp120 in saline. Guinea pig immunization study was performed using 50 µg dose gp120 mixed with 1% NE and saline as diluent.

Immunization procedures. Balb/c mice were immunized with two, and on one occasion with three, intranasal (i.n.) administrations of gp120/NE formulation at 3 weeks apart. The immunizations were performed by slowly applying gp120/NE mixes (10 µl per nare) to the nares of Isoflurane anesthetized mice. During delivery animals were held in the inverted position until droplets were completely inhaled. In control groups, mice were immunized with gp120 in saline, and with either NE or saline alone. Intramuscular immunization (i.m.) was performed with two doses, 3 weeks apart, of 20 µg gp120 injected in 50 µl of either saline or 1% NE. Hartley guinea pigs (3 animals per group) were anesthetized with Ketamine injection (40 mg/kg) and immunized intranasally with two i.n. administrations of gp120/NE mix (50 µl per nare) at 3 weeks apart.

Collection of blood, bronchial alveolar lavage, vaginal washes and splenocyte samples. Blood samples were obtained either from the saphenous vein, at various time points during the course of the experiment, or by cardiac puncture from euthanized premonitory mice. Serum was obtained from coagulated blood (30-60 minutes at room temperature) by centrifugation at 1500 g for 5 minutes. Collected serum samples were heat inactivated at 56°C for 1 hour and stored at -20°C until analyzed.

Mouse bronchial alveolar lavage fluid (BAL) was obtained from animals euthanized

by inhalation of Isoflurane. The lung was infused twice with 0.5 ml of PBS with 10 μ M DTT and 0.5 mg/ml aprotinin and approximately 1 ml of aspirate was recovered. BAL samples were stored at -20°C until analyzed.

Vaginal wash samples were collected from anesthetized mice by infusion of vaginal cavities with 100 μ l of PBS with 10 μ M DTT and 0.5 mg/ml aprotinin. The samples were centrifuged at 10,000 $\times$ g for 5 minutes at 4°C, and the supernatants were stored at -20°C until analyzed.

Murine splenocytes were mechanically isolated from the spleens to obtain single cell suspension in PBS. The red blood cells (RBC) were removed by lysis with ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA), and the remaining cells were washed twice in PBS. For antigen-specific proliferation or cytokine expression assays, splenocytes (2-4 $\times$ 10⁶ cells/ml) were resuspended in RPMI 1640 medium, supplemented with 2% FBS, L-glutamine, and penicillin/streptomycin (100 U/ml and 100 μ g/ml).

Determination of anti-gp120 IgG and IgA antibodies. Mouse anti-gp120-specific IgG and IgA levels were determined by ELISA. Microtiter plates (MAXISORP; NALGE NUNC International, Rochester, NY) were coated with 5 μ g/ml (100 μ l) of either gp120_{BaL} or gp120_{SF162} serotype envelope protein in the coating buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4°C. After the protein solution was removed, plates were blocked for 30 minutes at 37°C with PBS-1% dry milk solution. The blocking solution was aspirated and plates were used immediately or stored sealed at 4°C until needed. Serum and BAL samples were serially diluted in 0.1% BSA in PBS, and 100 μ l/well aliquots were incubated in gp120 coated plates for 1 hour at 37°C. Plates were washed three times with PBS-0.05% Tween 20, followed by 1 hour incubation with either anti-mouse IgG or anti-mouse IgA alkaline phosphatase (AP)-conjugated antibodies, then washed three times and incubated with AP substrate SIGMAFAST (SIGMA, St. Louis, MO) according to the manufacturer's protocol. Spectrophotometric readouts were performed using the SPECTRA MAX 340 ELISA reader (MOLECULAR DEVICES, Sunnyvale, CA) at 405 nm and reference wavelength of 690 nm. Endpoint antibody titers were defined as the last reciprocal serial serum dilution at which the absorption at 405 nm was greater than two times absorbance above negative control. Guinea pig anti-gp120 IgG was determined by the same method, except that rabbit anti-guinea pig IgG alkaline phosphatase (AP)-conjugate was used for detection (ROCKLAND). Antibody

concentrations are presented as the mean +/- standard deviation (s.d.) of endpoint titers.

HIV-1 single-round neutralization assay. An eight strain panel of clade B HIV-1 used in this study contained the laboratory strains BaL, SF162 and MN, and primary HIV-1 isolates SS1196.01, BG1168.01, QH0692.42, 3988.25 and 5768.04 (Li 05). Virus neutralization was measured as a function of the reduction in luciferase reporter gene expression after a single round of virus infection in TZM-bl cells as described (See, e.g., Montefiori, editor. Evaluating neutralizing antibodies against HIV, SIV and SHIV in luciferase reporter gene assays. New York, NY: John Wiley & Sons, 2004). The TZM-bl cells are engineered to express CD4 and CCR5 and contain integrated reporter genes for firefly luciferase and *E. coli* β -galactosidase under control of an HIV-1 LTR. Primary HIV-1 isolates (TCID₅₀, 100 to 200) were incubated with serial dilutions of sera for 1 hour at 37°C. Subsequently virus/serum mixtures were added to the 96-well flat-bottom culture plate containing adherent TZM-bl cells. Control contained cells plus virus (virus control), and cells only (background control). Bioluminescence was measured after 48 hours using BRIGHT GLO substrate solution as described by the supplier (PROMEGA, Madison, WI). Neutralization titers (NT₅₀) are the dilutions at which relative light units (RLU) were reduced by 50% compared to those of virus control wells after subtraction of background RLUs.

Proliferation assay. The proliferation of mouse splenocytes was measured by an assay of 5-bromo-2-deoxyuridine (BrdU) incorporation using a commercially available labeling and detection kit (Cell Proliferation ELISA, ROCHE Molecular Biochemicals, Mannheim, Germany). To assess antigen specific proliferation, cells (2×10^6 cell/ml) were incubated in medium alone and the presence of gp120_{BaL} (5 μ g/ml), or as control with a PHA-P (2 μ g/ml), for 48 hours and then pulsed with BrdU for 24 hours. Cell proliferation was measured according to the manufacturer's instructions using SPECTRA MAX 340 ELISA Reader (MOLECULAR DEVICES, Sunnyvale, CA) at 370 nm and reference wavelength of 492 nm.

Analysis of cytokine expression *in vitro*. Mouse splenocytes were seeded at 4×10^6 cells/ml (RPMI 1640, 2% FBS) and incubated with either gp120_{BaL}, gp120_{SF162} (5 μ g/ml) or with V3 loop peptide (20 nM) for 72 hours at 37°C. Cell culture supernatants were harvested and analyzed for the presence of cytokines. The cytokine assays were performed using QUANTIKINE ELISA kits (R&D SYSTEMS, Inc., Minneapolis, MN) according to

the manufacturer's instructions:

Statistical Analysis. Statistical analysis of the results was performed using ANOVA, and Student's T-test for the determination of the *p value*.

Example 18

Nasal immunization with recombinant HIV gp120 protein mixed with nanoemulsion induces potent IgG response in serum.

In order to determine whether NE has an adjuvant activity in the mucosal immunization with a recombinant HIVgp120 protein, Balb/c mice were intranasally (i.n.) immunized with either gp120_{BaL} or gp120_{SF162} serotype of antigen. Effect of NE concentration was assessed using 20 µg of gp120_{BaL} in saline or mixed with a 0.1%, 0.5% and 1% range of NE concentrations. Blood was collected at 6 weeks after two immunizations and at 12 weeks after three immunizations and analyzed for gp120-specific antibodies by ELISA. All mice immunized with either of gp120_{BaL}/NE preparations were seropositive after only two immunizations. The anti-gp120_{BaL} IgG response showed concentration-dependent effect of NE, with lowest titers in gp120_{BaL}/0.1% NE and highest in gp120_{BaL}/1% NE immunization groups (mean titers of 1.3×10^4 and 2.6×10^5 , respectively). Mice immunized with gp120_{BaL}/saline did not have detectable anti-gp120_{BaL} antibodies. Serum anti-gp120 IgG titers after i.n. immunization with either 0.5% or 1% NE were comparable, or even higher, than antibody response after two i.m. injections with gp120_{BaL} in saline or mixed with 1% NE. A third i.n. immunization did not significantly increase antibody titers in either of the immunization groups (See Figure 14A). Thus, the present invention provides that only two i.n. administrations of gp120_{BaL} with NE adjuvant are required to mount a potent systemic IgG response in mice.

NE is sufficient for robust mucosal adjuvation. NE-produced immune responses were compared with the effects of known immunostimulants, unmethylated CpG ODN and MPL A. Mice were i.n. immunized with 20 µg gp120_{SF162} mixed with 1% NE (gp120_{SF162}/NE) and compared to immunization with antigen mixed with either CpG ODN (gp120_{SF162}/CpG) or with MPL A (gp120_{SF162}/MPL A). In order to investigate the effect of combining the NE with immunostimulants, mice were immunized with a gp120_{SF162}/NE and additionally with either CpG (gp120_{SF162}/NE+CpG) or MPL A (gp120_{SF162}/NE+MPL A). Similar to immunization with gp120_{BaL}, mice immunized with gp120_{SF162}/NE responded

with high anti-gp120_{SF162} IgG titers. Combination of NE with MPL A (but not with CpG) resulted in a modest increase in mean antibody titer (2 to 3 fold over immunization with gp120_{SF162}/NE alone), however the difference was not statistically significant ($p > 0.05$). In contrast, immunizations with antigen mixed with either CpG or MPL A alone produced only weak immune response (See Figure 14B).

Example 19

Antibodies generated against one serotype of gp120 cross-react with other gp120 serotypes.

Experiments conducted during the development of the present invention determined that i.n. immunization with either serotype of gp120 protein produced highly cross-reacting IgG antibodies. For example, the IgG antibody raised against either gp120_{BaL} or gp120_{SF162} cross-reacted with a heterologous serotype with activity that was comparable with binding to autologous envelope protein (See Figures 14C and 14D). Thus, the present invention provides that mucosal immunization with either serotype of gp120 can induce comparable immune responses. Thus, in some embodiments, NE adjuvant can produce a repertoire of IgG capable of recognizing both variable and conserved epitopes of the gp120 immunogen (e.g., that participate in protective immunity against various types of HIV-1 (See, e.g., Mascola, Curr Mol Med 2003;3(3):209-16)).

Example 20

Nasal administration of gp120/NE generates anti-gp120 specific IgA antibodies detectable in bronchial and vaginal mucosal surfaces.

BAL fluids, vaginal washes and sera were analyzed for the assessment of mucosal response. Mice i.n. immunized with gp120_{SF162}/NE had significant levels of gp120_{SF162}-specific secretory IgA and IgG antibodies in BAL fluid (See Figures 15A and 15B). Similar to serum, both IgA and IgG antibodies demonstrated cross-reactivity with heterologous gp120_{BaL} immunogen. Anti-gp120_{BaL} IgA antibodies were also detected in serum and distant mucosal sites (e.g., as measured in vaginal wash samples (See Figure 15C)). Immunization with either type of gp120 in saline failed to produce mucosal IgA and IgG responses detectable in the BAL, serum, and vaginal secretions. Thus, the present invention provides that significant mucosal responses, both locally (e.g., in bronchial mucosa) and in

distant sites (e.g., vaginal secretions), can be induced in response to i.n. immunization with antigen (e.g., gp120) delivered with NE adjuvant.

Example 21

Cell mediated immune responses.

Cellular immune responses were assessed *in vitro* by antigen-specific T-cell proliferation assays as well as characterization of T helper-type cytokine production. Antigen specific proliferative responses were detected in re-stimulated splenic lymphocytes from animals immunized with the gp120_{BaL}/NE but were absent in either mice immunized with gp120_{BaL}/saline or with control animals (treated with saline or NE alone) (See Figure 16A). Intranasal immunization with gp120_{BaL}/NE produced strong cell-mediated immune responses as measured by splenic IFN- γ production (See Figure 16B). *In vitro* stimulation with either gp120_{BaL} or gp120_{SF162} serotypes produced high IFN- γ responses to both autologous (BaL) and heterologous (SF162) types of gp120. A substantial induction of IFN- γ was also obtained with an oligopeptide fragment of the V3 loop, indicating the presence of CTLs specific for the dominant epitope involved in virus binding and neutralization (See, e.g., Kwong et al., Nature 1998;393(6686):648-59; Takahashi et al., Science 1992;255(5042):333-6). Antigen-specific induction of IFN- γ and the lack of detectable IL-4 expression evidences Th1 polarization of the cellular immune response. No significant cytokine expression was detected in splenocytes from control mice or from mice immunized with gp120_{BaL} in saline.

Example 22

Immunization with gp120/NE induces HIV-1 neutralizing antibodies.

In order to characterize potential neutralizing activity of gp120-specific antibodies induced by mucosal immunization, guinea pigs were administered with two doses of gp120_{SF162} mixed with 1% NE. Immunization produced significant, albeit varied, levels of serum anti-gp120 IgG antibodies in individual animals (See Figure 17A). As observed in mice, the guinea pig anti-gp120 IgG cross-reacted with heterologous gp120 immunogen. Immune sera from guinea pigs were tested for neutralizing activity against HIV-1. The breadth of the neutralizing response was evaluated in a panel of 8 viruses, including 3 laboratory strains and 5 primary HIV isolates. The highest neutralizing titer (NT₅₀) toward autologous M-tropic strain of HIV_{SF162} was detected in serum from the most responsive

animal ($NT_{50} = 225$) (See Figure 17B). However, significant neutralizing activity ($NT_{50} > 50$) was also detected in two other animals, despite much lower anti-gp120 IgG levels.

Neutralization of heterologous M-tropic strain HIV_{BaL} was comparable in all guinea pigs with NT_{50} greater than 50. No neutralization was observed with laboratory strain of T-tropic HIV_{MN} virus. All five primary HIV isolates tested were effectively neutralized with sera from vaccinated guinea pigs. Neutralizing activity for the primary HIV isolates was comparable with both laboratory strains. The NT_{50} values for BG1168.1, SS1196.11 and 3988.25 ranged from 50 to 100 depending on the serum. The isolates QH0692.42 and 5768.4 were effectively neutralized with NT_{50} values greater than 100.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described compositions and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the present invention.

CLAIMS

We claim:

1. A method of inducing an immune response to an orthopox virus in a subject comprising:
 - a) providing a composition comprising a nanoemulsion and an immunogen, wherein said immunogen comprises an orthopox virus inactivated by said nanoemulsion; and
 - b) administering said composition to said subject under conditions such that said subject generates an immune response to said orthopox virus.
2. The method of claim 1, wherein said administering comprises contacting a mucosal surface of said subject with said composition.
3. The method of claim 2, wherein said mucosal surface comprises nasal mucosa.
4. The method of claim 1, wherein said inducing an immune response induces immunity to said orthopox virus in said subject.
5. The method of claim 4, wherein said immunity comprises systemic immunity.
6. The method of claim 4, wherein said immunity comprises mucosal immunity.
7. The method of claim 1, wherein said immune response comprises increased expression of IFN- γ in said subject.
8. The method of claim 1, wherein said immune response comprises a systemic IgG response to said inactivated orthopox virus.
9. The method of claim 1, wherein said immune response comprises a mucosal IgA response to said inactivated orthopox virus.

10. The method of claim 1, wherein said orthopox virus inactivated by said nanoemulsion is administered to said subject under conditions such that between 10 and 10^3 pfu of said inactivated virus is present in a dose administered to said subject.
11. The method of claim 1, wherein a 10% nanoemulsion solution is utilized to inactivate said vaccinia virus.
12. The method of claim 1, wherein said nanoemulsion comprises W₂₀₅EC.
13. The method of claim 1, wherein said immunity protects said subject from displaying signs or symptoms of disease caused by said orthopox virus.
14. The method of claim 1, wherein said immunity protects said subject from challenge with a subsequent exposure to live orthopox virus.
15. The method of claim 1, wherein said composition further comprises an adjuvant.
16. The method of claim 1, wherein said subject is a human.
17. The method of claim 1, wherein said orthopox virus is vaccinia virus.
18. The method of claim 13, wherein said subject is protected from displaying signs and symptoms of smallpox.
19. A composition for stimulating an immune response comprising a nanoemulsion and an orthopox virus inactivated by said nanoemulsion, wherein said composition is configured to induce immunity to said orthopox virus in a subject.
20. The composition of claim 19, wherein said nanoemulsion comprises W₂₀₅EC.
21. The composition of claim 19, wherein said composition provides said subject between 10 and 10^3 pfu of said inactivated virus when administered to said subject.

22. The composition of claim 19, wherein a dose of said composition administered to said subject comprises a 1% nanoemulsion solution.
23. The composition of claim 19, wherein said inactivated orthopox virus is heat stable in said nanoemulsion.
24. The composition of claim 23, wherein said orthopox virus is stable for greater than four weeks in said nanoemulsion.
25. The composition of claim 19, wherein said composition is diluted prior to administration to a subject.
26. The composition of claim 23, wherein said subject is a human.
27. The composition of claim 19, wherein said immunity is systemic immunity.
28. The composition of claim 19, wherein said immunity is mucosal immunity.
29. The composition of claim 19, wherein said composition further comprises an adjuvant.
30. The composition of claim 19, wherein said orthopox virus is vaccinia virus.
31. The composition of claim 19, wherein said orthopox virus is selected from the group consisting of variola virus, cowpox, monkeypox, gergilpox, and camelpox.
32. A kit comprising a composition for stimulating an immune response comprising a nanoemulsion and an orthopox virus inactivated by said nanoemulsion, wherein said composition is configured to induce immunity to said orthopox virus in a subject, and instructions for administering said composition.
33. The kit of claim 32, wherein said orthopox virus is vaccinia virus.

34. The kit of claim 32, wherein said orthopox virus is selected from the group consisting of variola virus, cowpox, monkeypox, gerbilpox, and camelpox.
35. A method of inducing an immune response to *B. anthracis* in a subject comprising:
- providing a composition comprising a nanoemulsion and an immunogen, wherein said immunogen comprises recombinant protective antigen (rPA) of *B. anthracis*; and
 - administering said composition to said subject under conditions such that said subject generates an immune response to said *B. anthracis*.
36. The method of claim 35, wherein said administering comprises contacting a mucosal surface of said subject with said composition.
37. The method of claim 36, wherein said mucosal surface comprises nasal mucosa.
38. The method of claim 35, wherein said inducing an immune response induces immunity to said *B. anthracis* in said subject.
39. The method of claim 38, wherein said immunity comprises systemic immunity.
40. The method of claim 38, wherein said immunity comprises mucosal immunity.
41. The method of claim 35, wherein said immune response comprises increased expression of IFN- γ in said subject.
42. The method of claim 35, wherein said immune response comprises a systemic IgG response to said *B. anthracis*.
43. The method of claim 35, wherein said immune response comprises a mucosal IgA response to said *B. anthracis*.
44. The method of claim 35, wherein said composition comprises between 25 and 75 μg of said rPA.

45. The method of claim 35, wherein said composition comprises a 10% nanoemulsion solution.
46. The method of claim 35, wherein said immunity protects said subject from displaying signs or symptoms of disease caused by *B. anthracis*.
47. The method of claim 35, wherein said immunity protects said subject from challenge with a subsequent exposure to live *B. anthracis*.
48. The method of claim 35, wherein said composition further comprises an adjuvant.
49. The method of claim 48, wherein said adjuvant comprises a CpG oligonucleotide.
50. The method of claim 35, wherein said subject is a human.
51. The method of claim 35, wherein said immunity protects said subject from displaying signs or symptoms of anthrax.
52. A composition for stimulating an immune response comprising a nanoemulsion and recombinant protective antigen of *B. anthracis*, wherein said composition is configured to induce immunity to said *B. anthracis* in a subject.
53. The composition of claim 52, wherein said nanoemulsion comprises W₂₀5EC.
54. The composition of claim 52, wherein said composition provides said subject between 25 and 75 µg of said recombinant protective antigen when administered to said subject.
55. The composition of claim 52, wherein a dose of said composition administered to said subject comprises a 1% nanoemulsion solution.
56. The composition of claim 52, wherein said recombinant protective antigen is heat

stable in said nanoemulsion.

57. The composition of claim 56, wherein said recombinant protective antigen is stable for greater than four weeks in said nanoemulsion.

58. The composition of claim 52, wherein said composition is diluted prior to administration to a subject.

59. The composition of claim 52, wherein said subject is a human.

60. The composition of claim 52, wherein said immunity is systemic immunity.

61. The composition of claim 52, wherein said immunity is mucosal immunity.

62. The composition of claim 52, wherein said composition further comprises an adjuvant.

63. The composition of claim 62, wherein said adjuvant comprises a CpG oligonucleotide.

64. A kit comprising a composition for stimulating an immune response comprising a nanoemulsion and recombinant protective antigen of *B. anthracis*, wherein said composition is configured to induce immunity to said *B. anthracis* in a subject, and instructions for administering said composition.

65. The kit of claim 64, further comprising a device for administering said composition.

66. The kit of claim 65, wherein said device is selected from the group consisting of a nasal applicator, a syringe, a nasal inhaler and a nasal mister.

67. A method of inducing an immune response to HIV in a subject comprising:
a) providing a composition comprising a nanoemulsion and an immunogen, wherein said immunogen comprises recombinant gp120; and

- b) administering said composition to said subject under conditions such that said subject generates an immune response to said HIV.
68. The method of claim 67, wherein said administering comprises contacting a mucosal surface of said subject with said composition.
69. The method of claim 68, wherein said mucosal surface comprises nasal mucosa.
70. The method of claim 67, wherein said inducing an immune response induces immunity to said HIV in said subject.
71. The method of claim 70, wherein said immunity comprises systemic immunity.
72. The method of claim 70, wherein said immunity comprises mucosal immunity.
73. The method of claim 67, wherein said immune response comprises increased expression of IFN- γ in said subject.
74. The method of claim 67, wherein said immune response comprises a systemic IgG response to said HIV.
75. The method of claim 67, wherein said immune response comprises a mucosal IgA response to said HIV.
76. The method of claim 67, wherein said composition comprises between 15 and 75 μg of said recombinant gp120.
77. The method of claim 67, wherein said composition comprises a 10% nanoemulsion solution.
78. The method of claim 67, wherein said immunity protects said subject from displaying signs or symptoms of disease caused by HIV.

79. The method of claim 67, wherein said immunity protects said subject from challenge with a subsequent exposure to live HIV.
80. The method of claim 67, wherein said composition further comprises an adjuvant.
81. The method of claim 80, wherein said adjuvant comprises a CpG oligonucleotide.
82. The method of claim 80, wherein said adjuvant comprises monophosphoryl lipid A.
83. The method of claim 67, wherein said subject is a human.
84. The method of claim 67, wherein said immunity protects said subject from displaying signs or symptoms of AIDS.
85. A composition for stimulating an immune response comprising a nanoemulsion and recombinant gp120, wherein said composition is configured to induce immunity to HIV in a subject.
86. The composition of claim 85, wherein said nanoemulsion comprises W₂₀5EC.
87. The composition of claim 85, wherein said nonemulsion comprises X8P.
88. The composition of claim 85, wherein said composition provides said subject between 15 and 75 µg of said recombinant gp120 when administered to said subject.
89. The composition of claim 85, wherein a dose of said composition administered to said subject comprises a 1% nanoemulsion solution.
90. The composition of claim 85, wherein said recombinant gp120 is heat stable in said nanoemulsion.
91. The composition of claim 85, wherein said composition is diluted prior to administration to a subject.

92. The composition of claim 85, wherein said subject is a human.
93. The composition of claim 85, wherein said immunity is systemic immunity.
94. The composition of claim 85, wherein said immunity is mucosal immunity.
95. The composition of claim 85, wherein said composition further comprises an adjuvant.
96. The composition of claim 95, wherein said adjuvant comprises a CpG oligonucleotide.
97. The composition of claim 95, wherein said adjuvant comprises monophosphoryl lipid A.
98. A kit comprising a composition for stimulating an immune response comprising a nanoemulsion and recombinant gp120, wherein said composition is configured to induce immunity to HIV in a subject, and instructions for administering said composition.
99. The kit of claim 98, further comprising a device for administering said composition.
100. The kit of claim 99, wherein said device is selected from the group consisting of a nasal applicator, a syringe, a nasal inhaler and a nasal mister.

FIGURE 1A

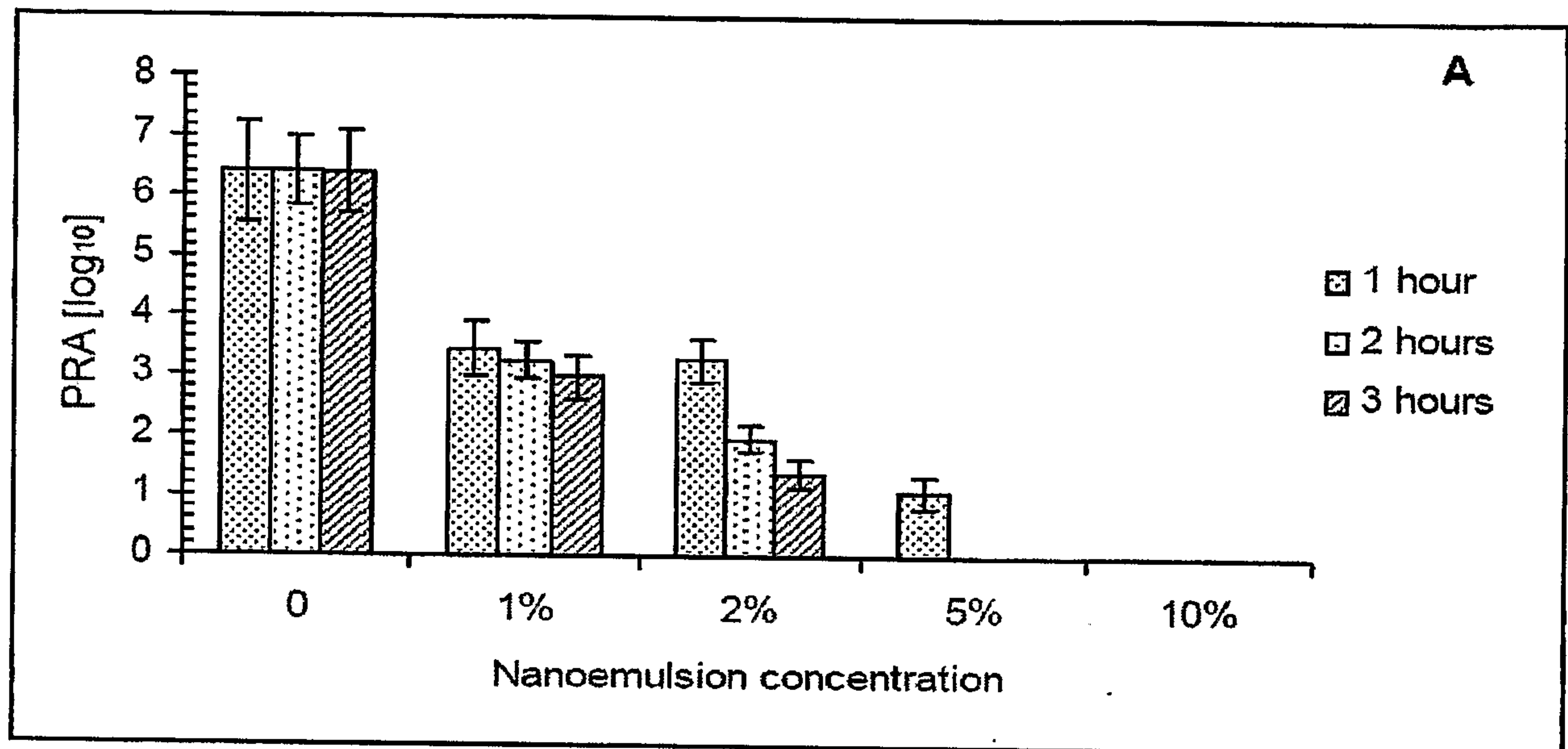


FIGURE 1B

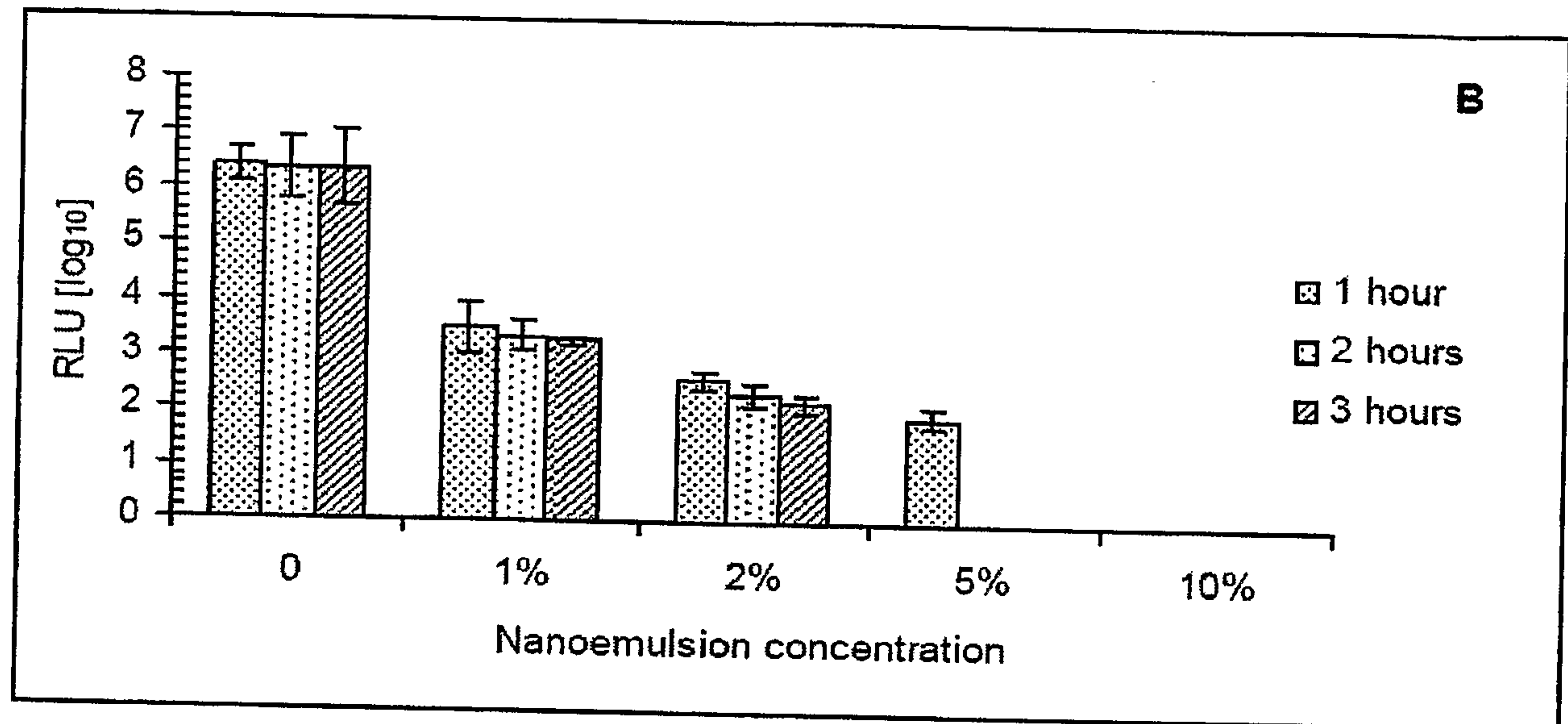


FIGURE 1C

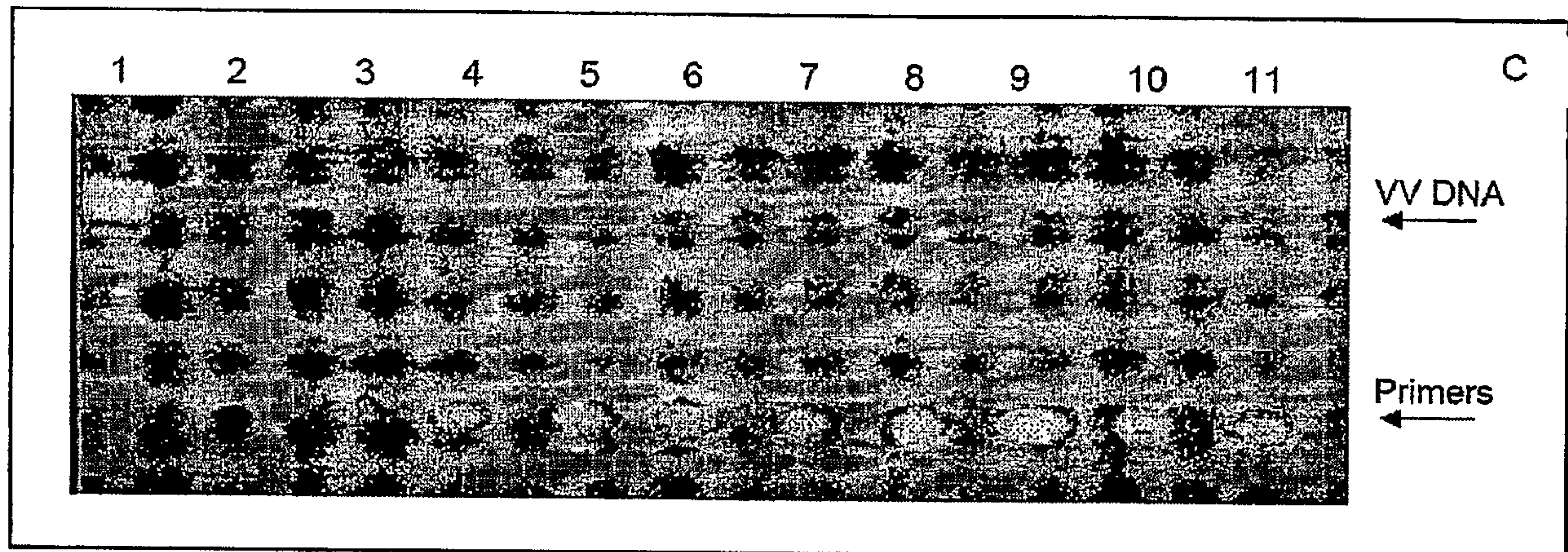


FIGURE 1D

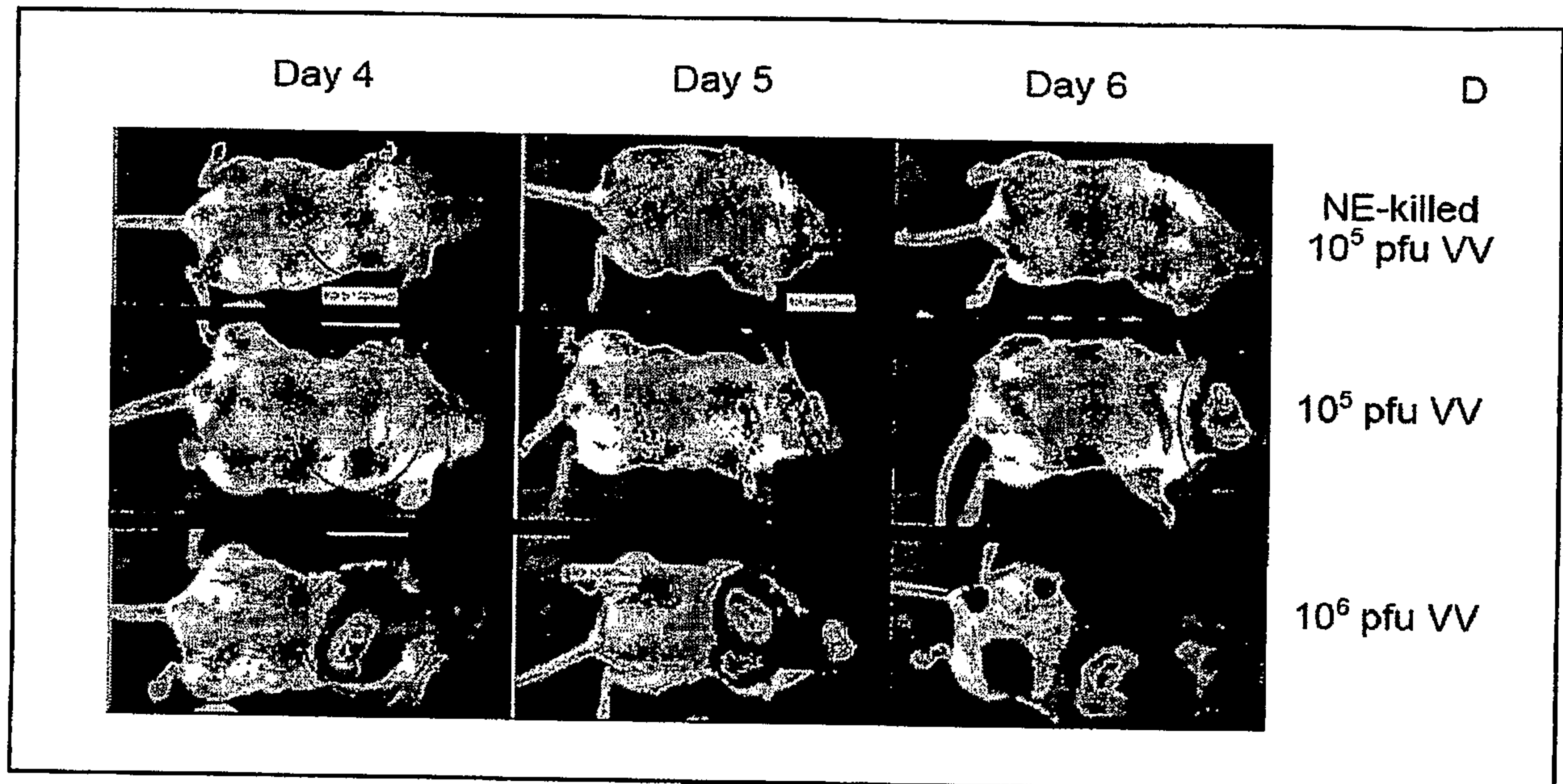


FIGURE 2A

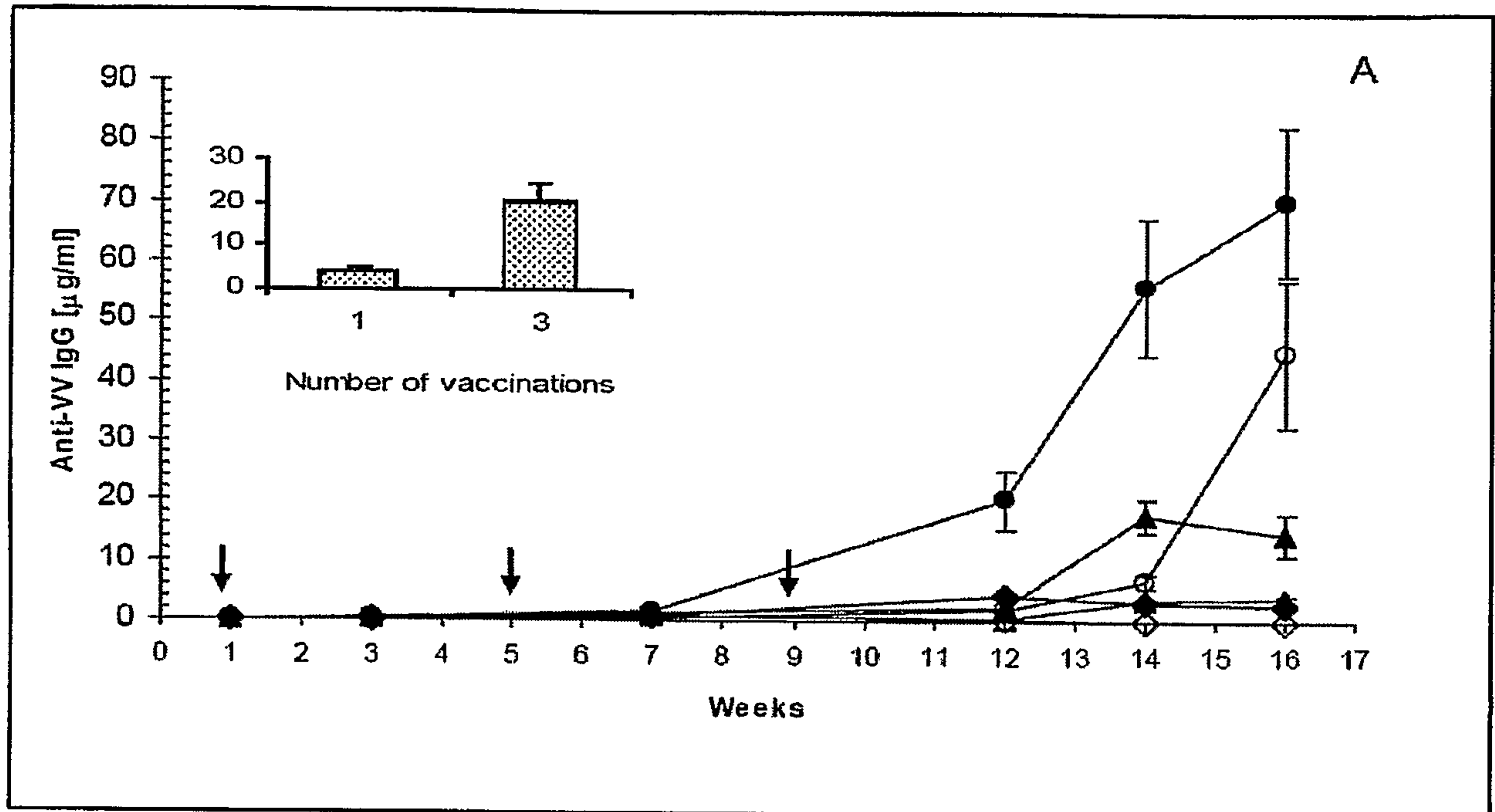


FIGURE 2B

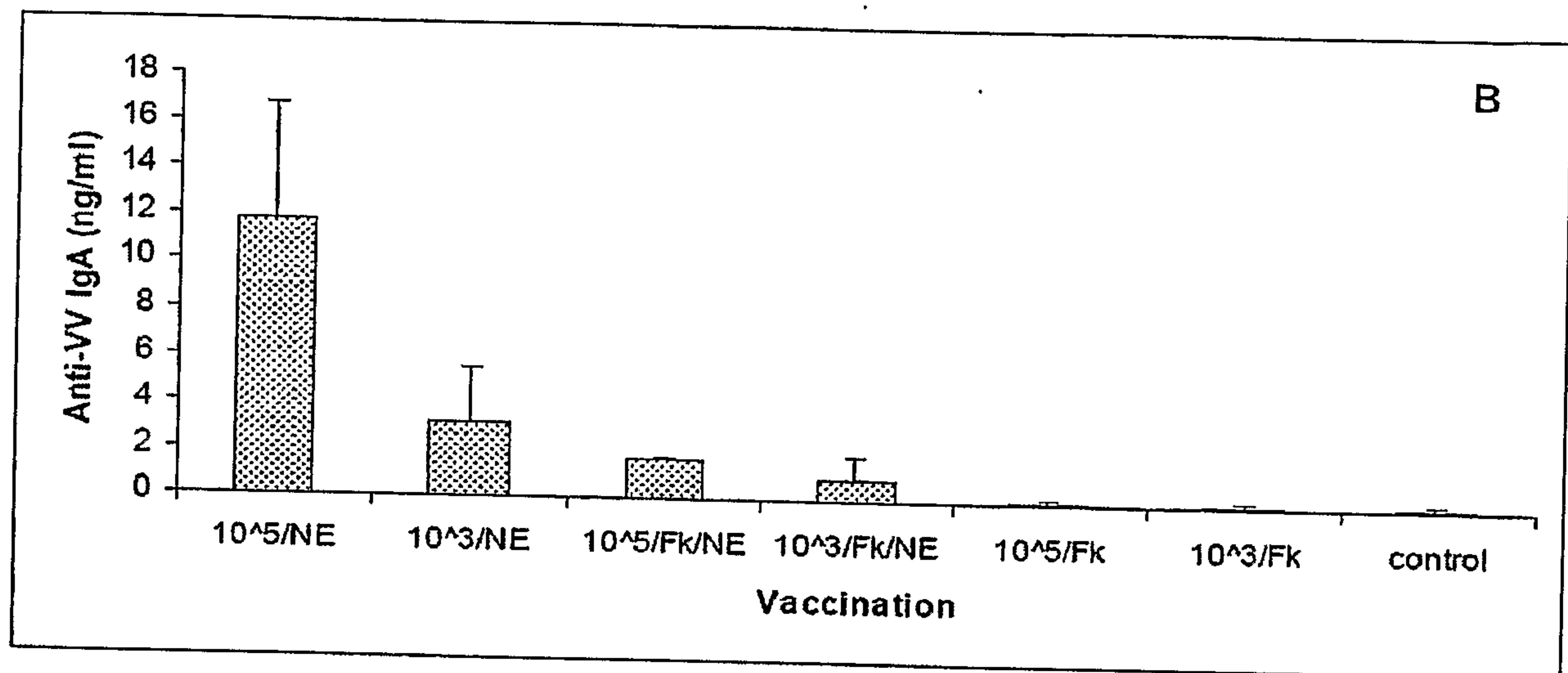


FIGURE 3

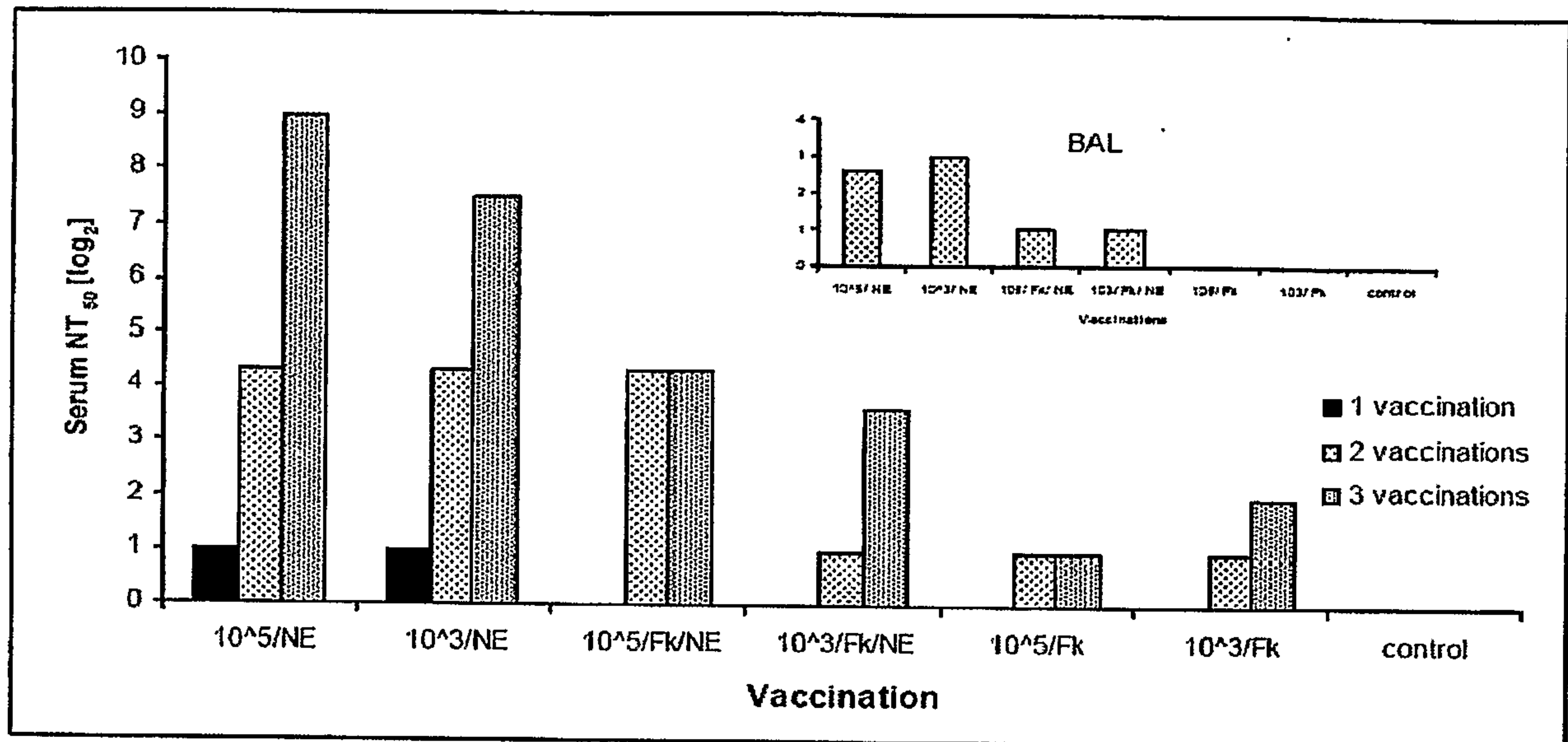


FIGURE 4

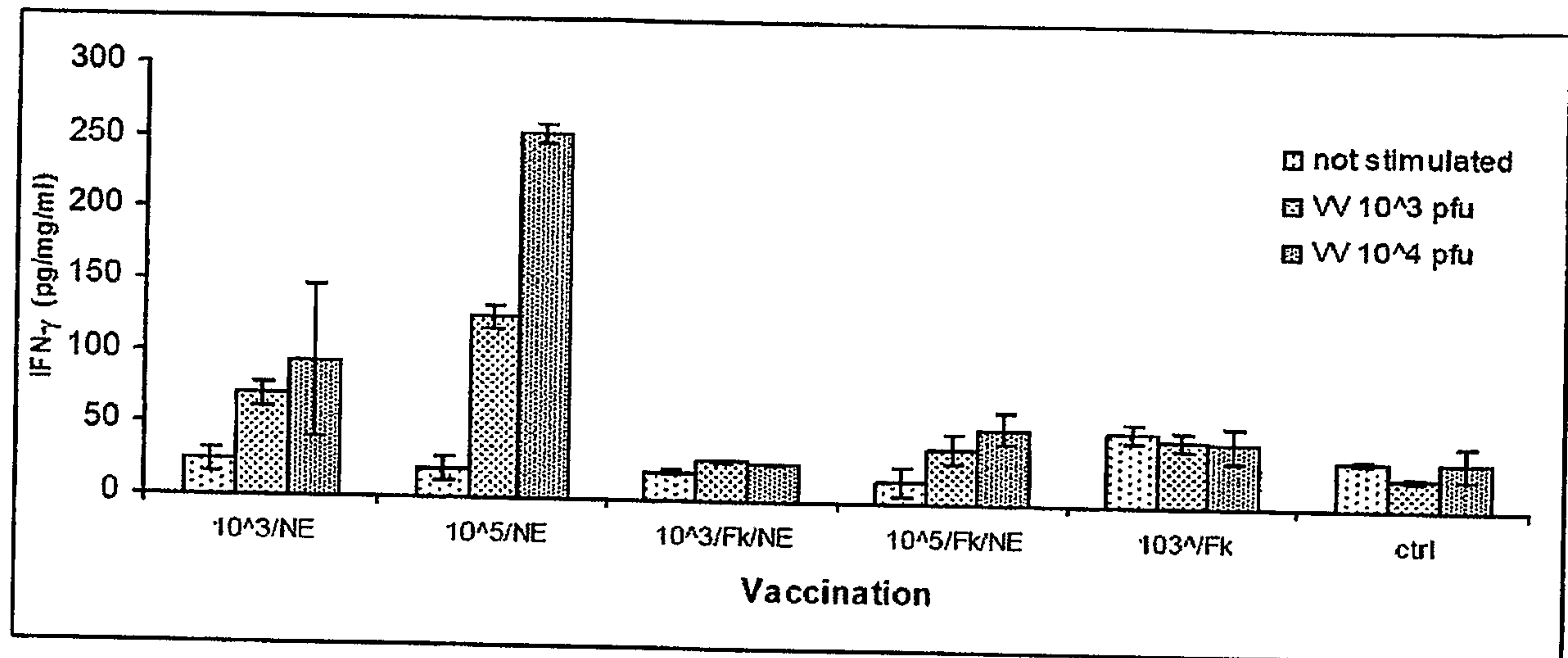


FIGURE 5A

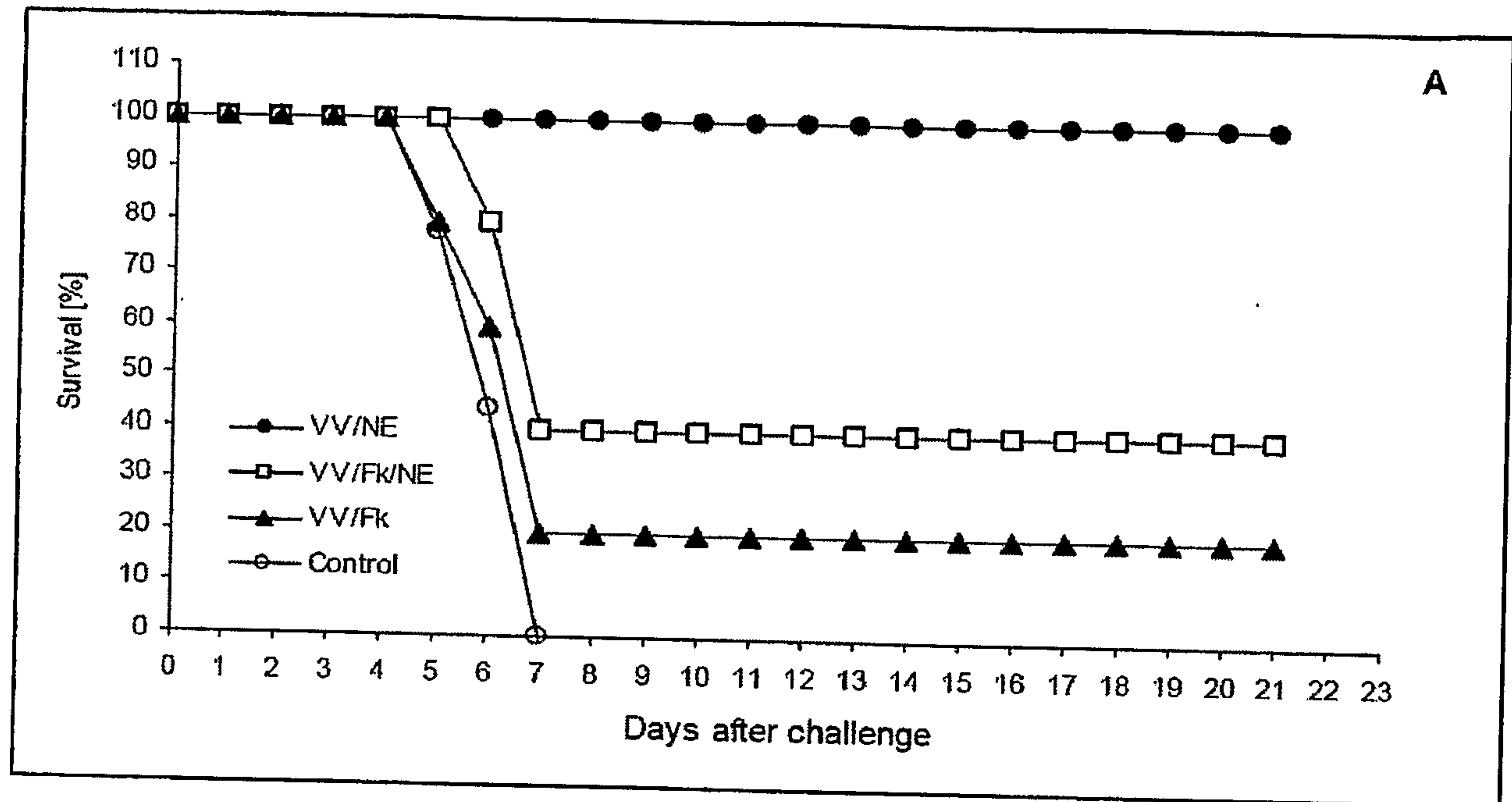


FIGURE 5B

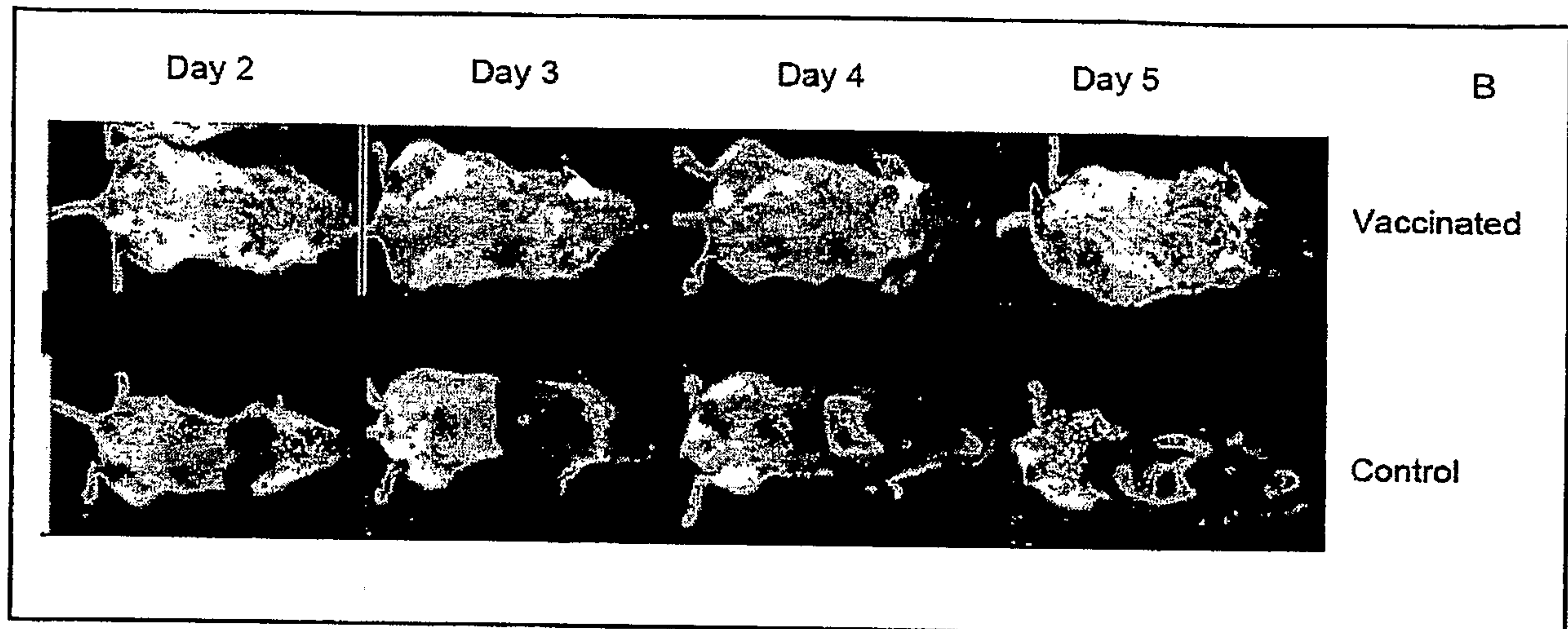


FIGURE 6

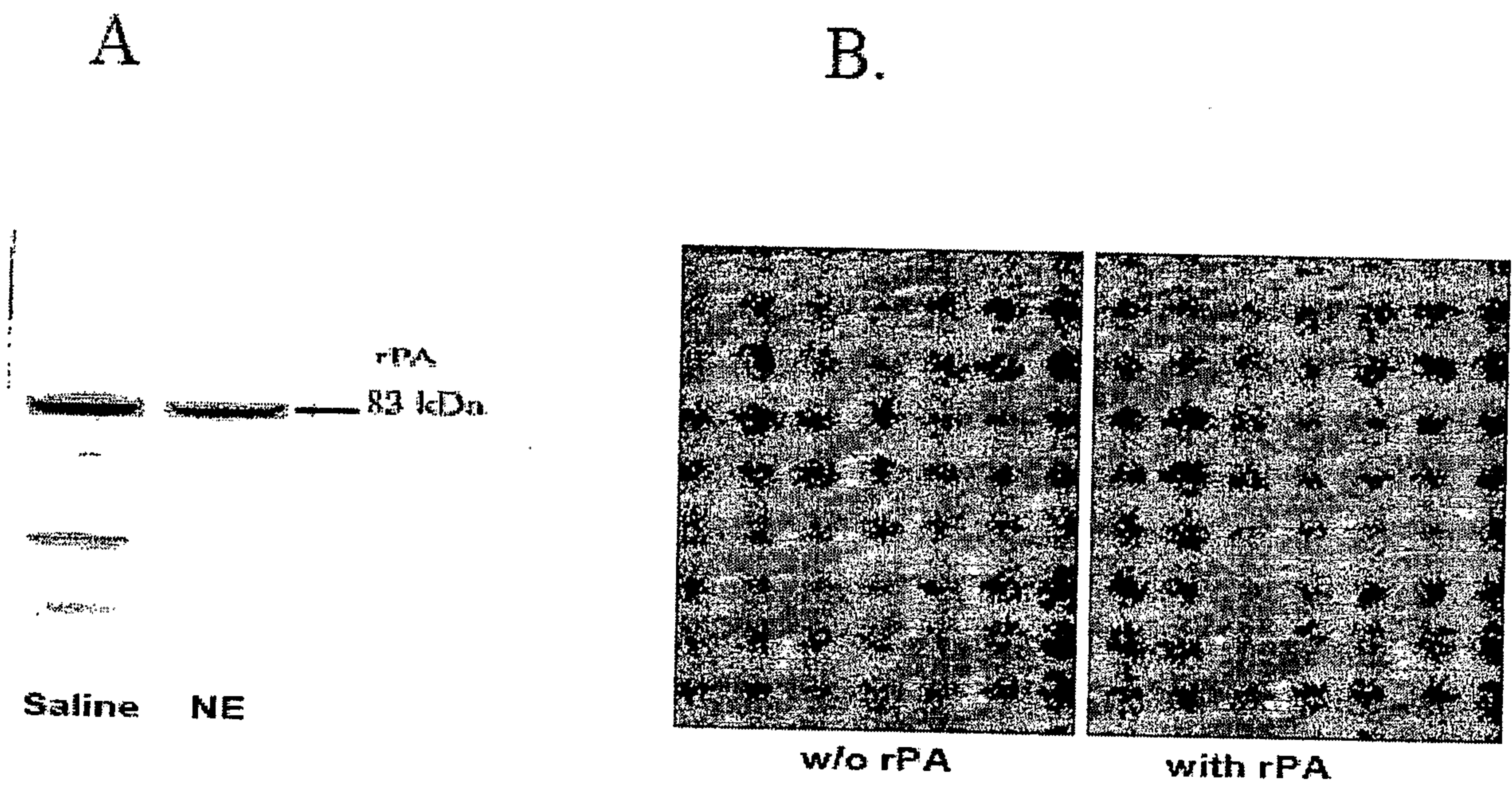


FIGURE 7

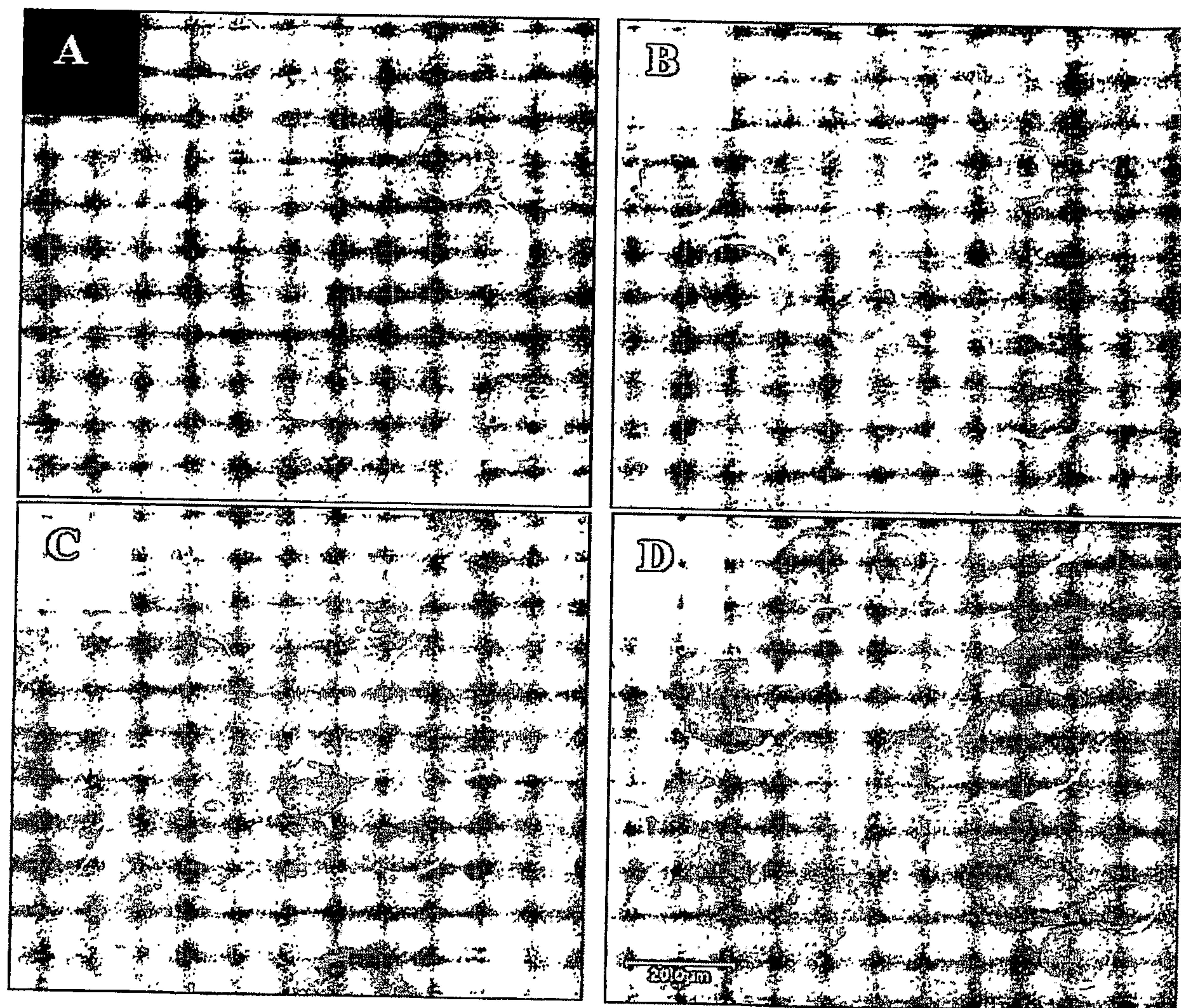


FIGURE 8A

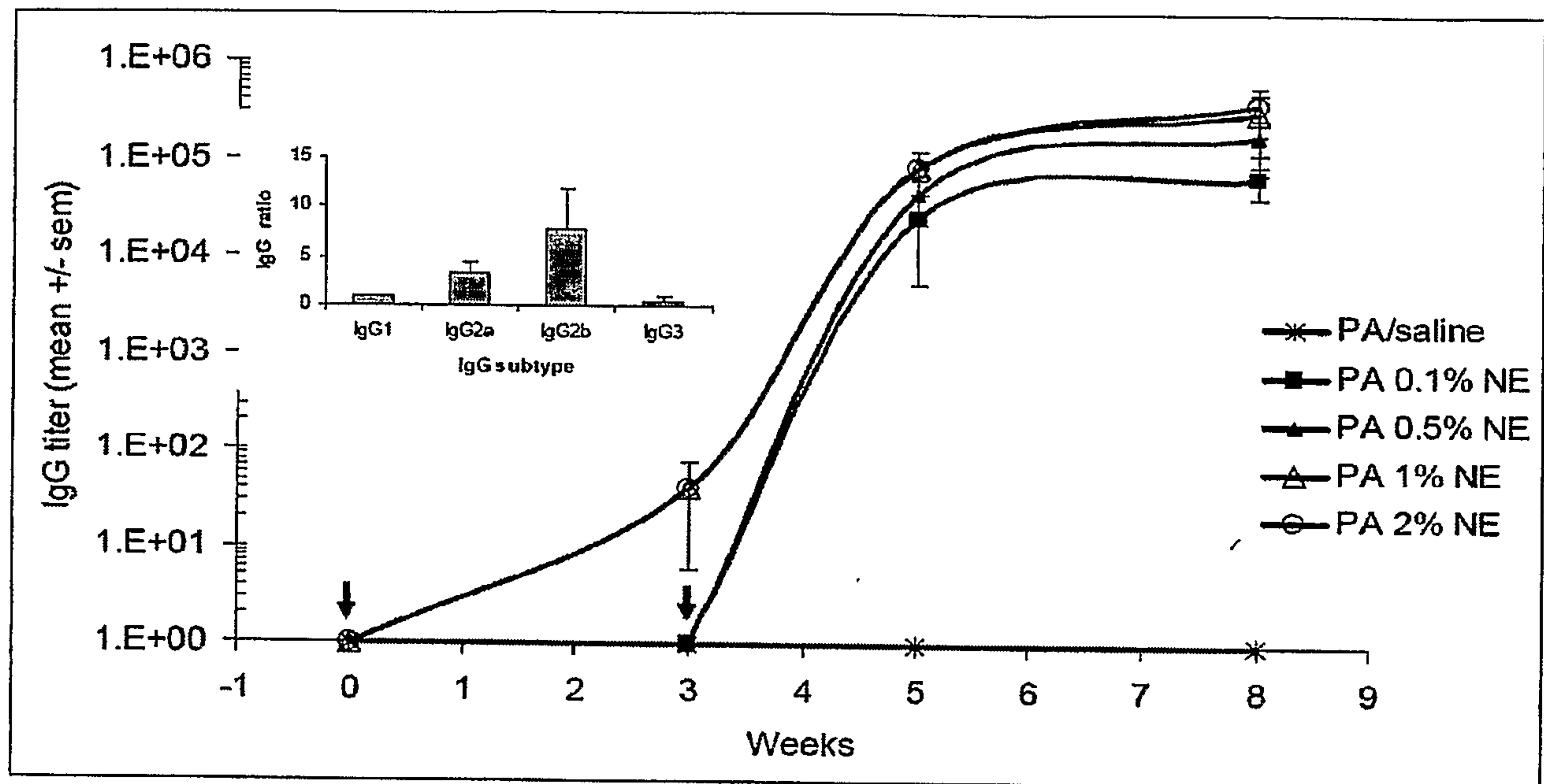


FIGURE 8B

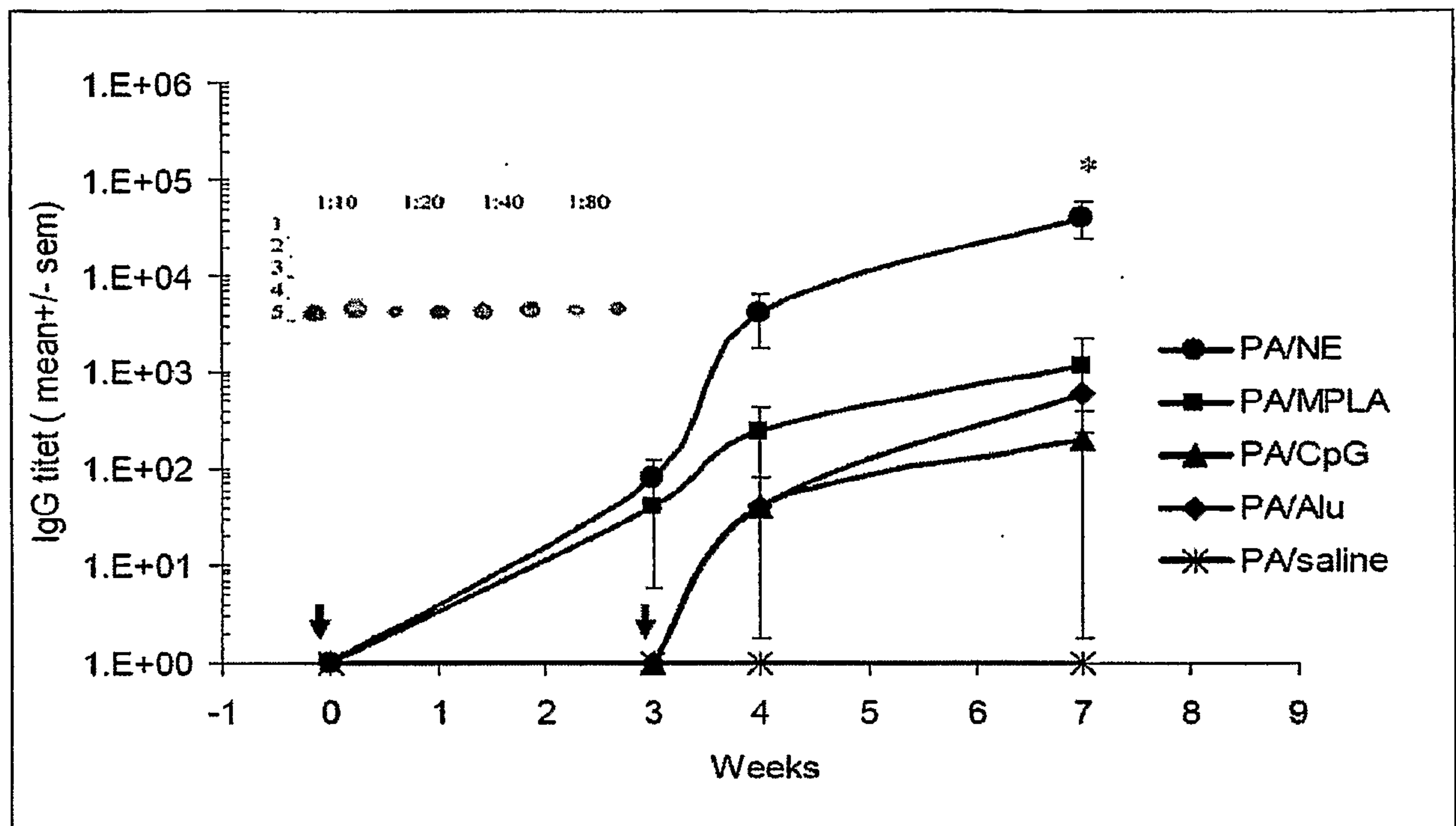


FIGURE 9

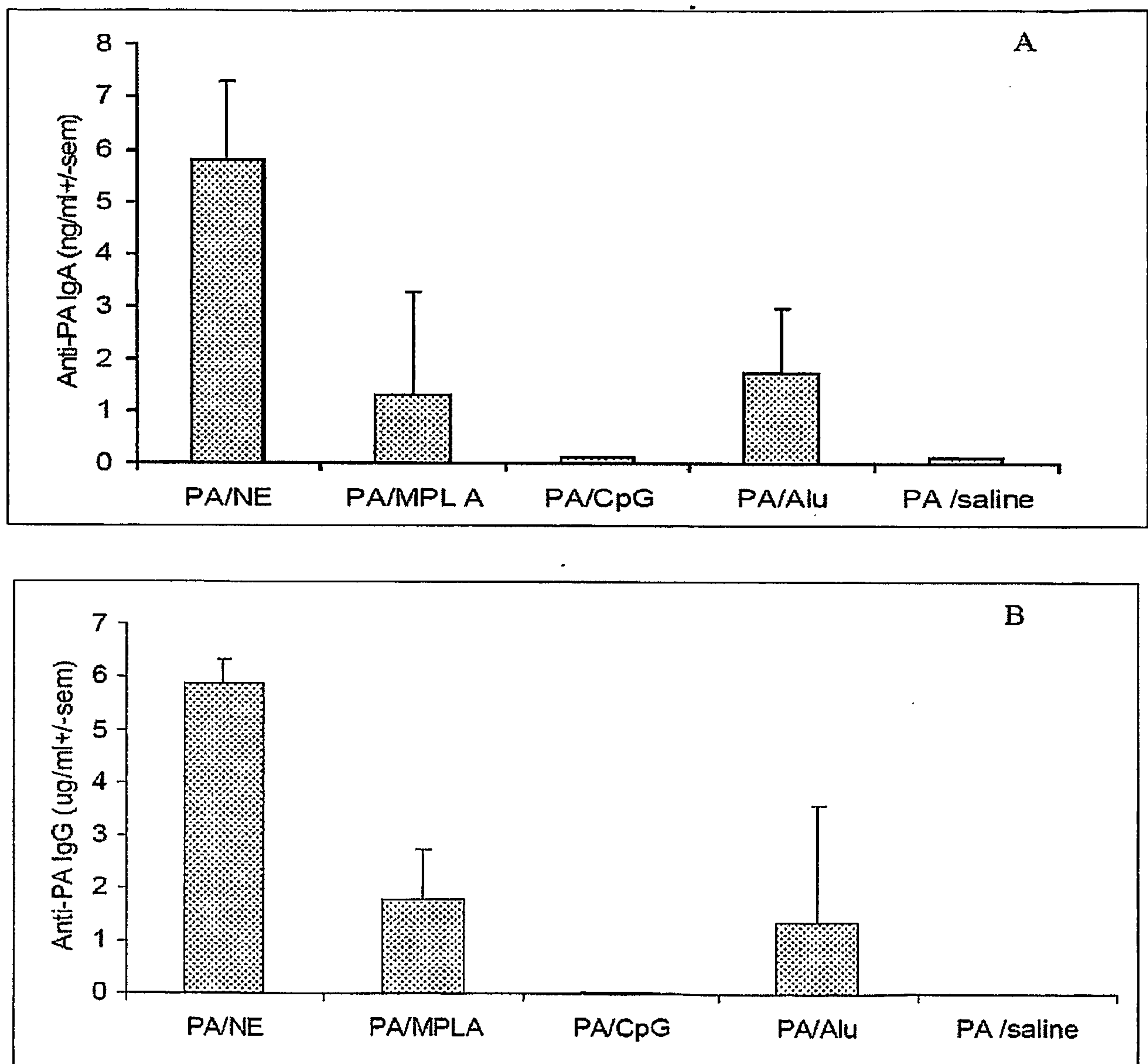


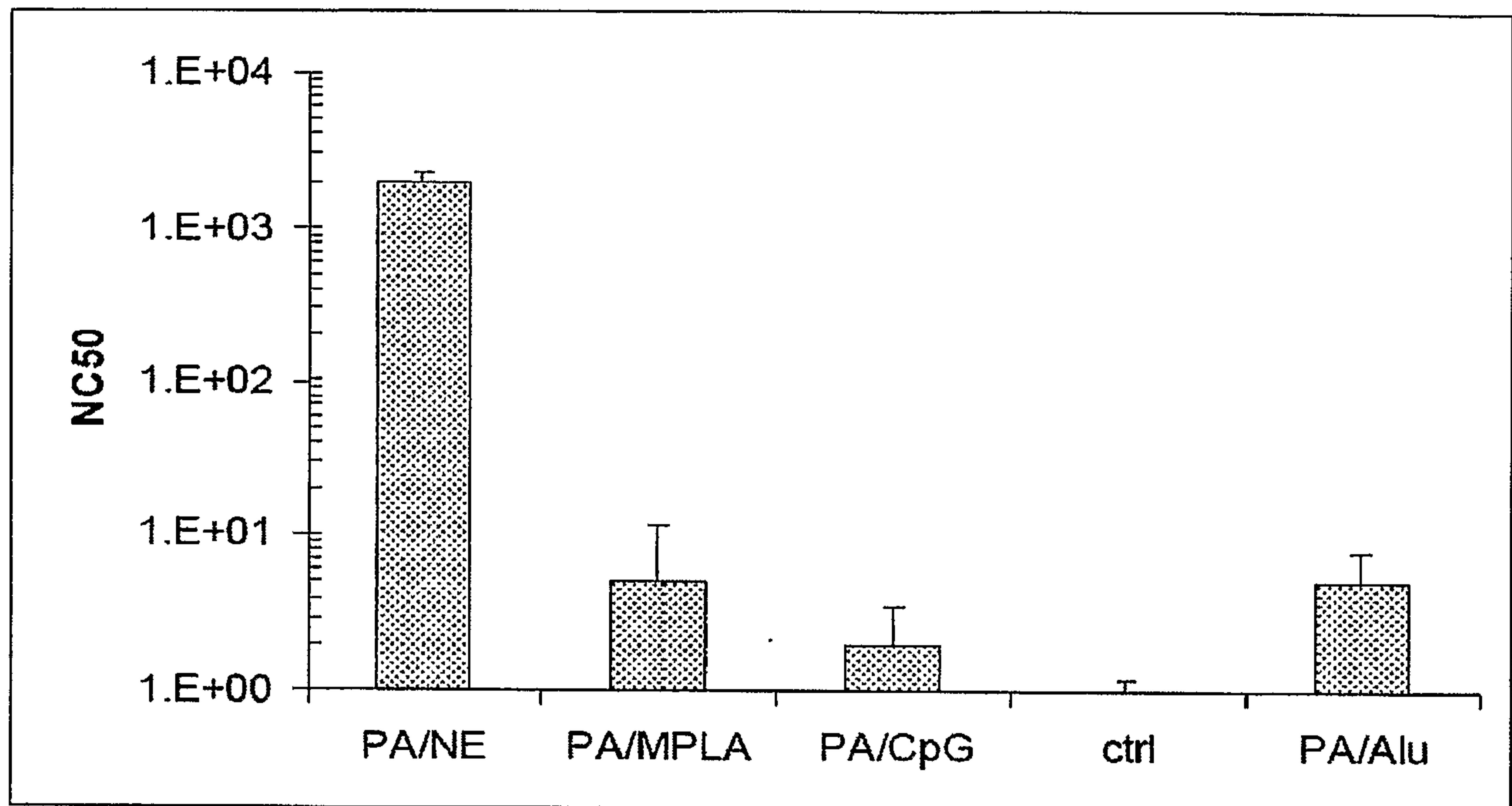
FIGURE 10

FIGURE 11

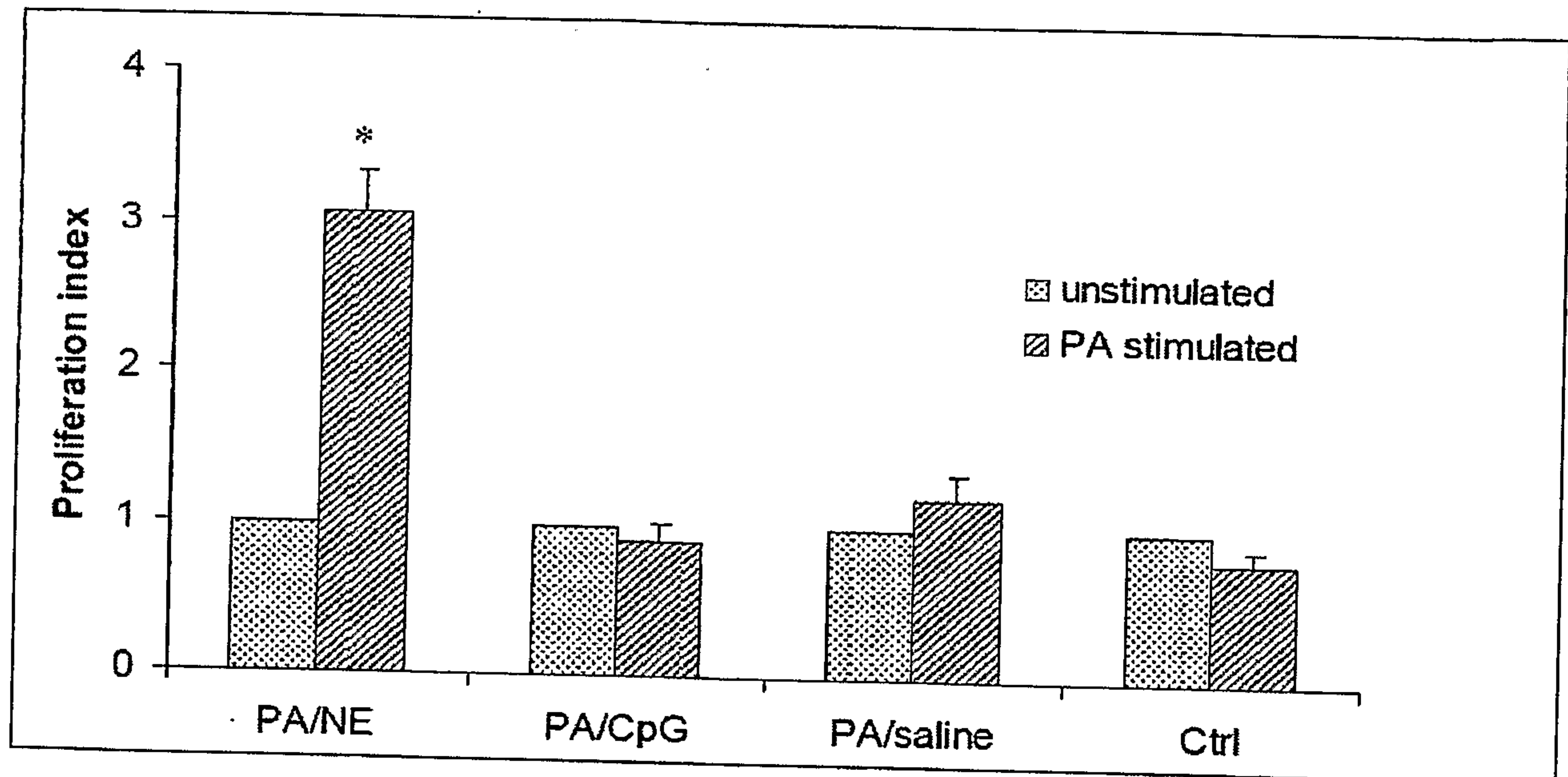


FIGURE 12A

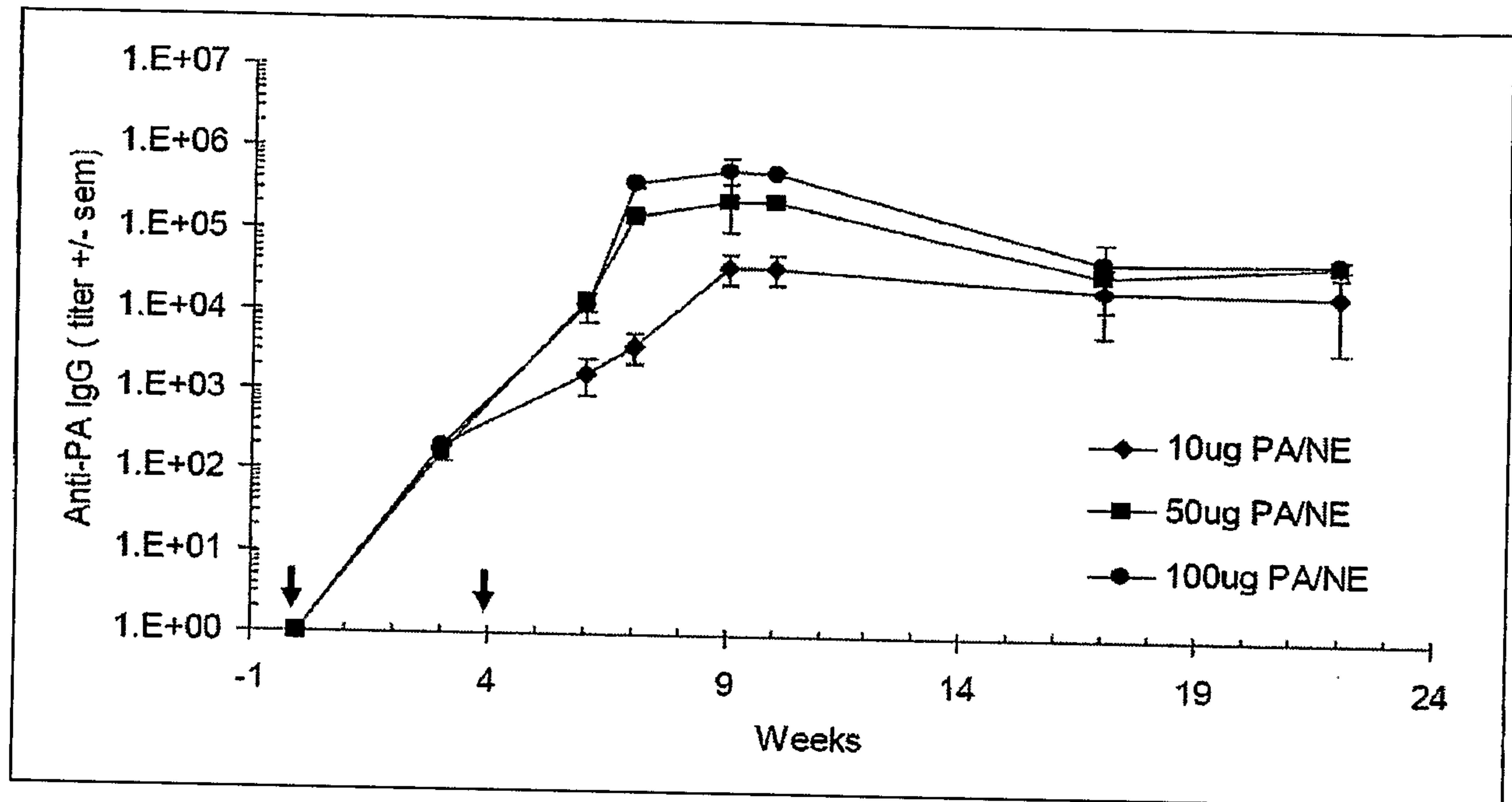


FIGURE 12B

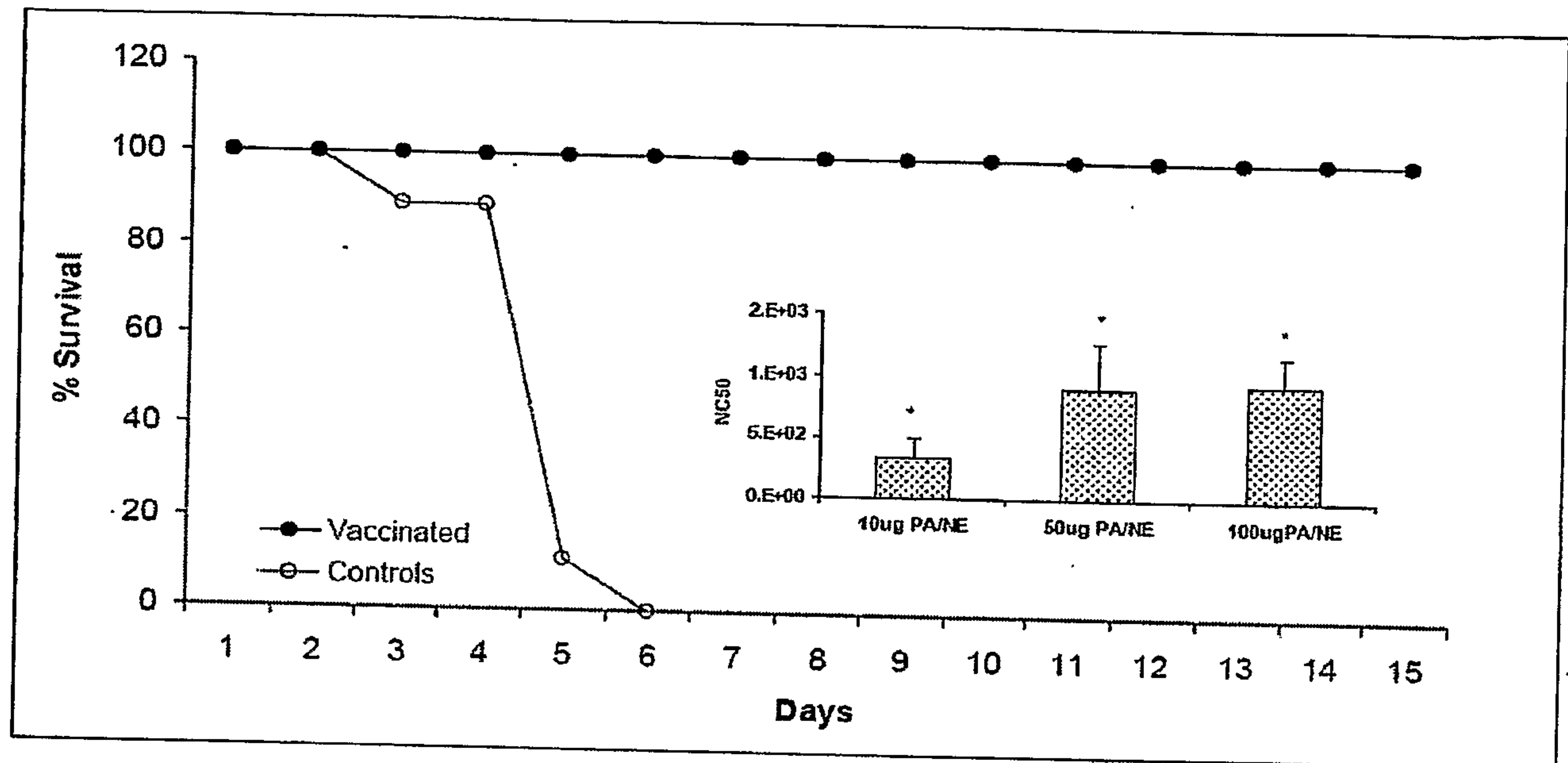


FIGURE 13A

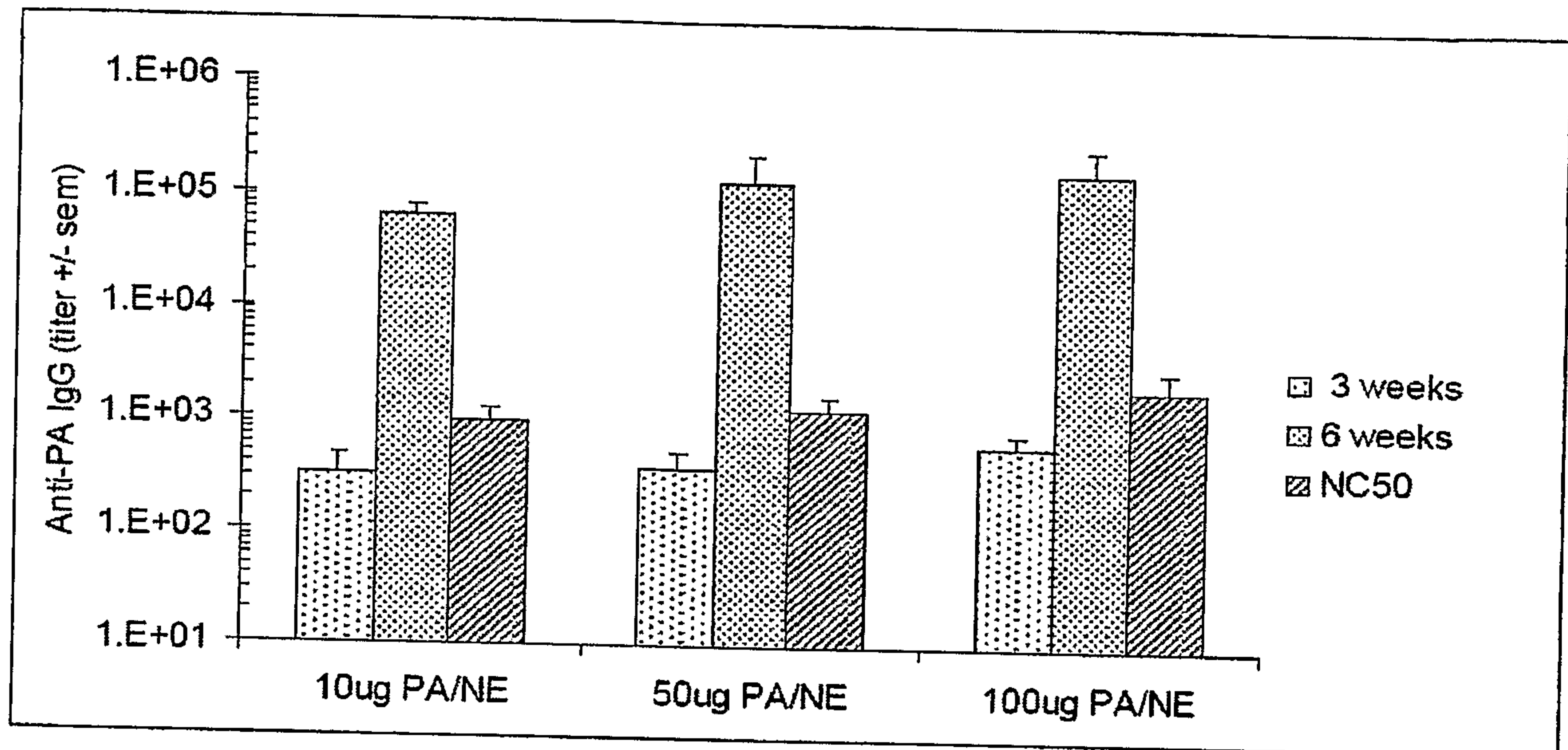


FIGURE 13B

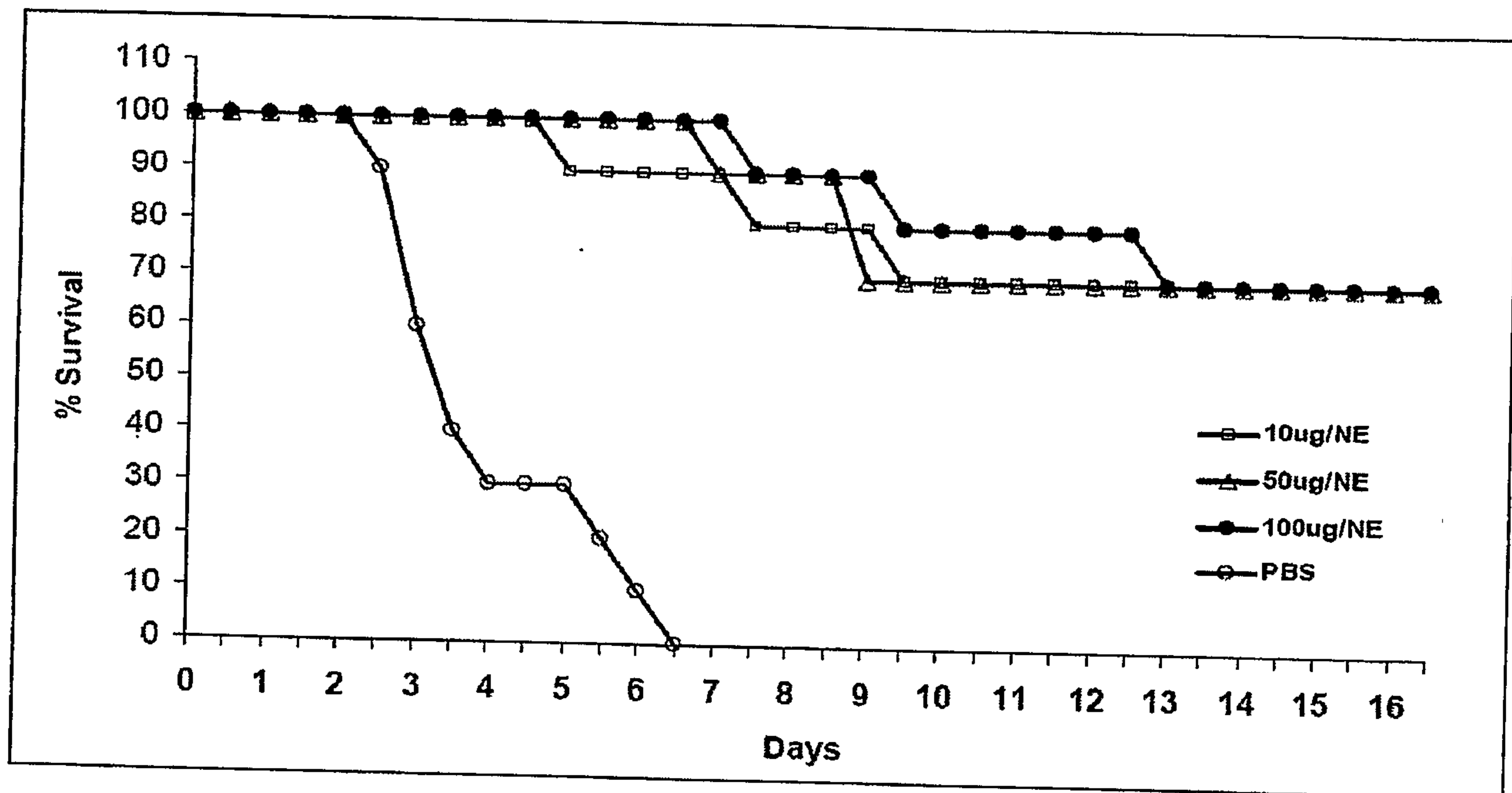


FIGURE 13C

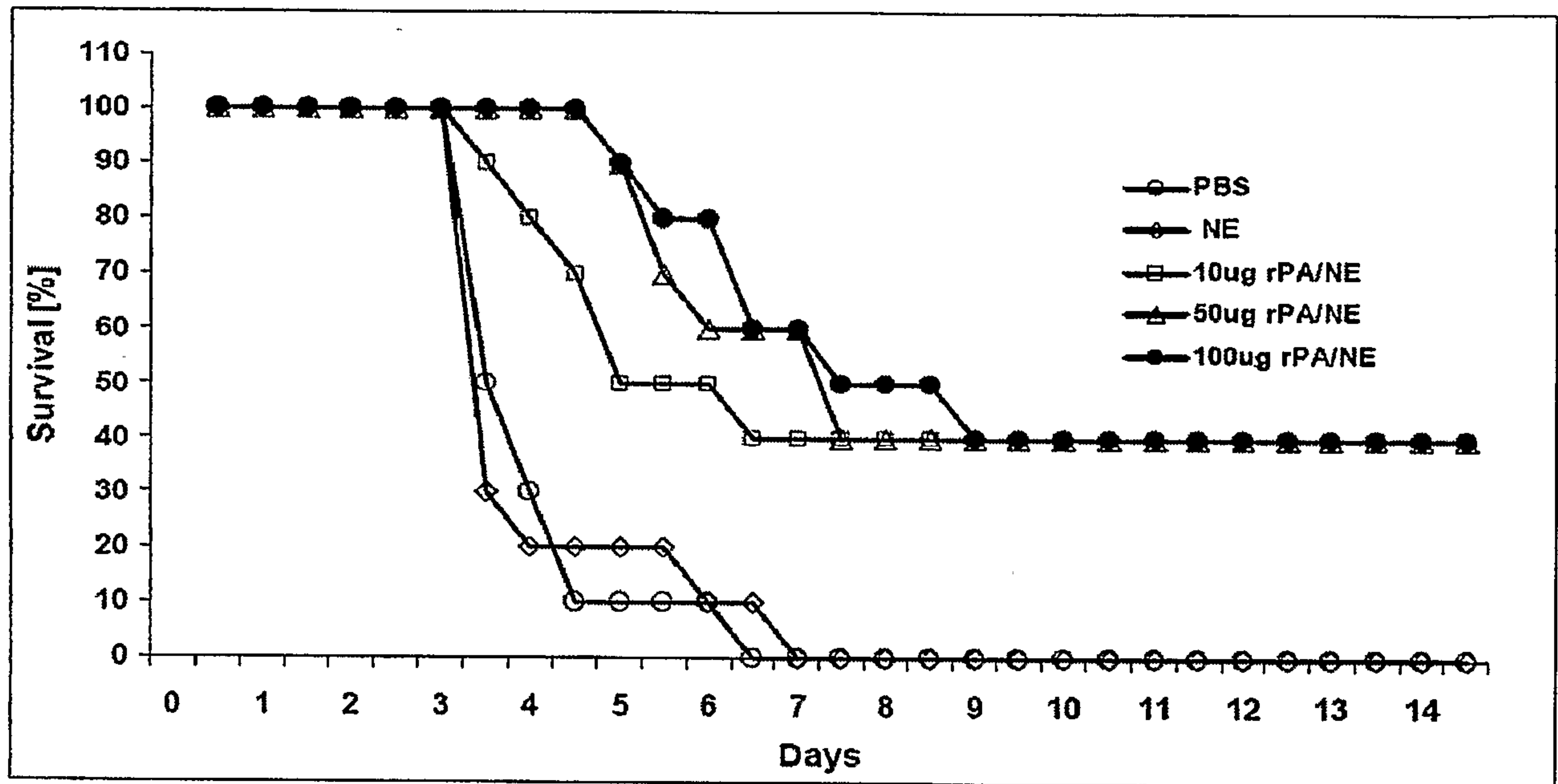


FIGURE 14A

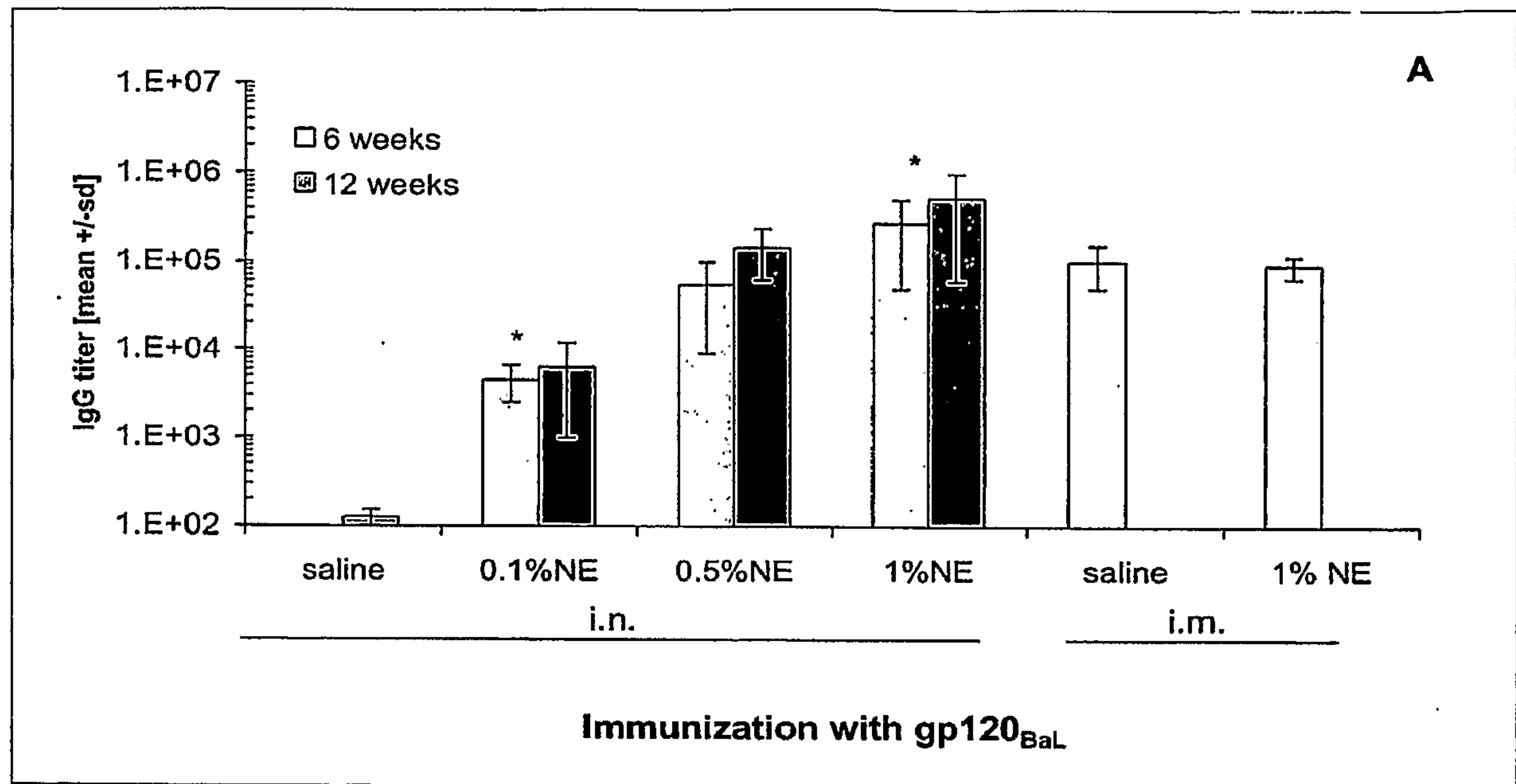


FIGURE 14B

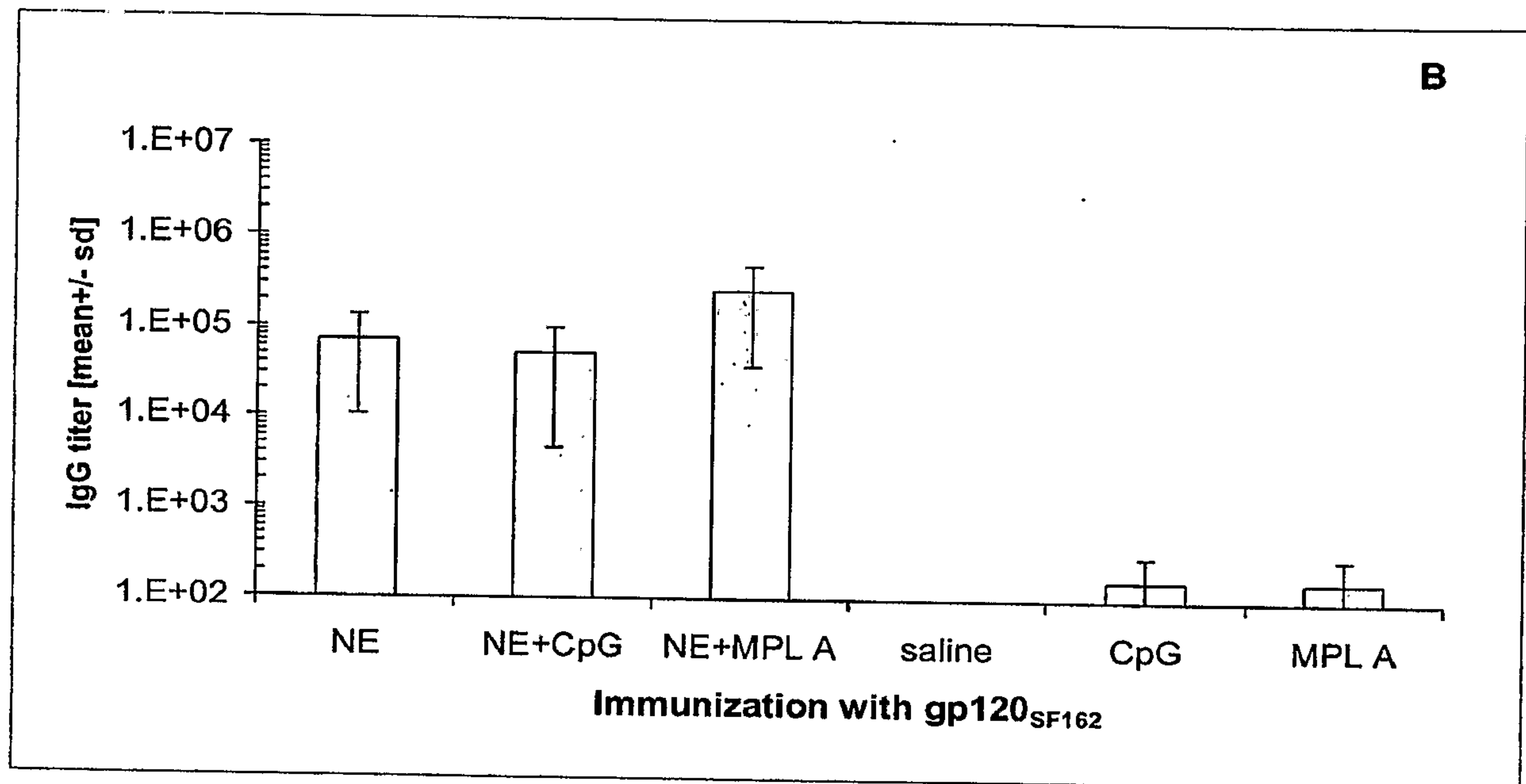


FIGURE 14C

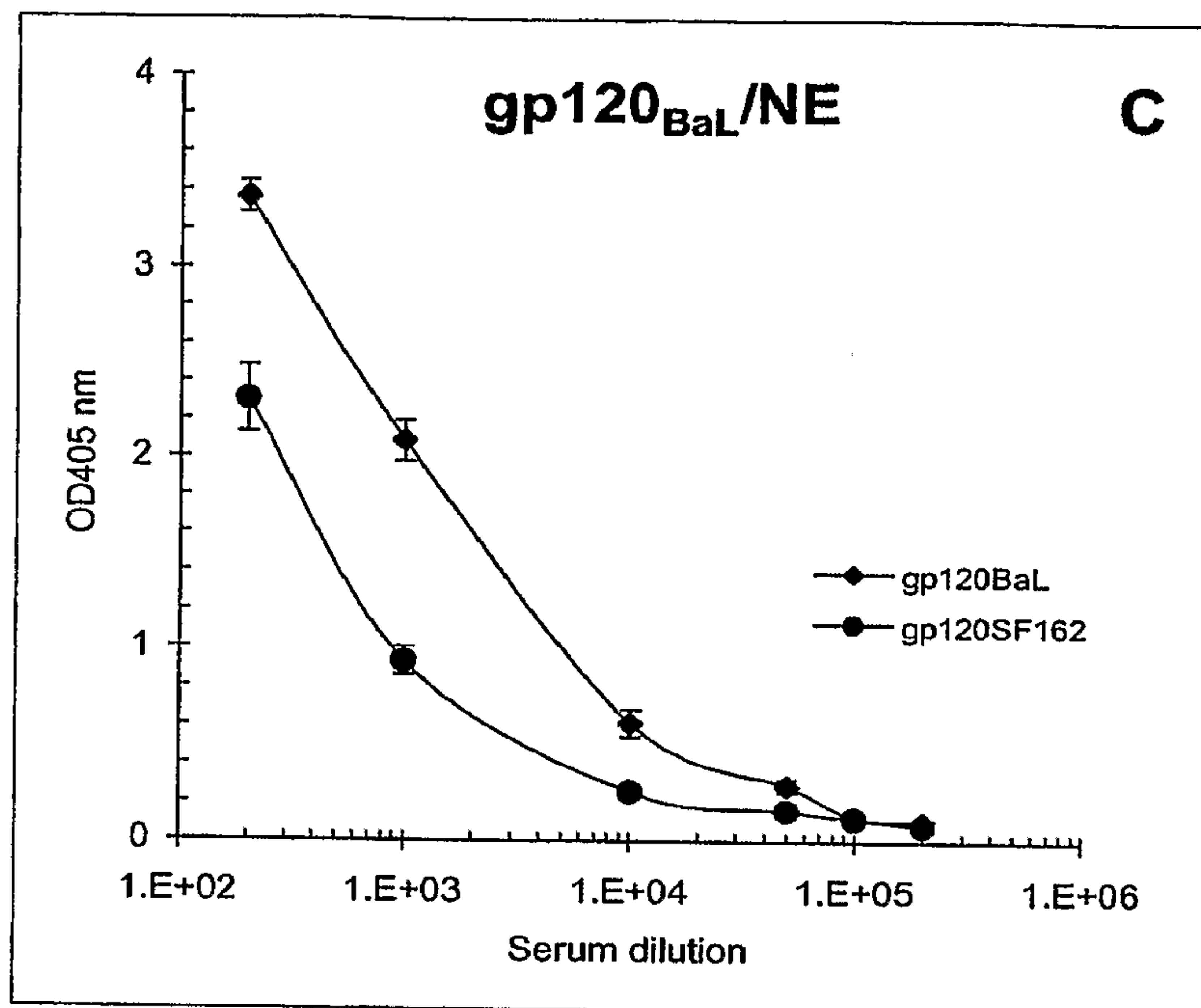


FIGURE 14D

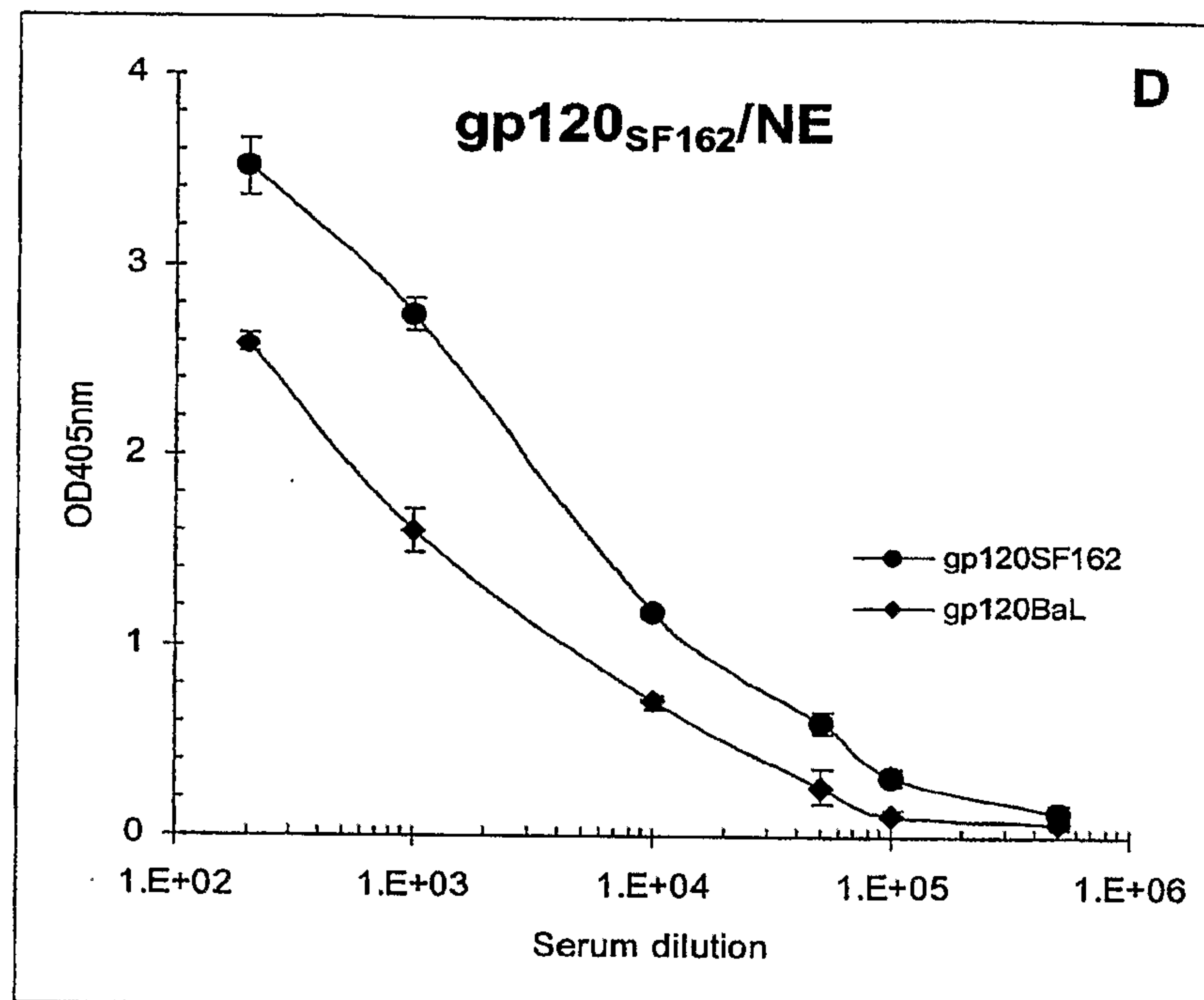


FIGURE 15A

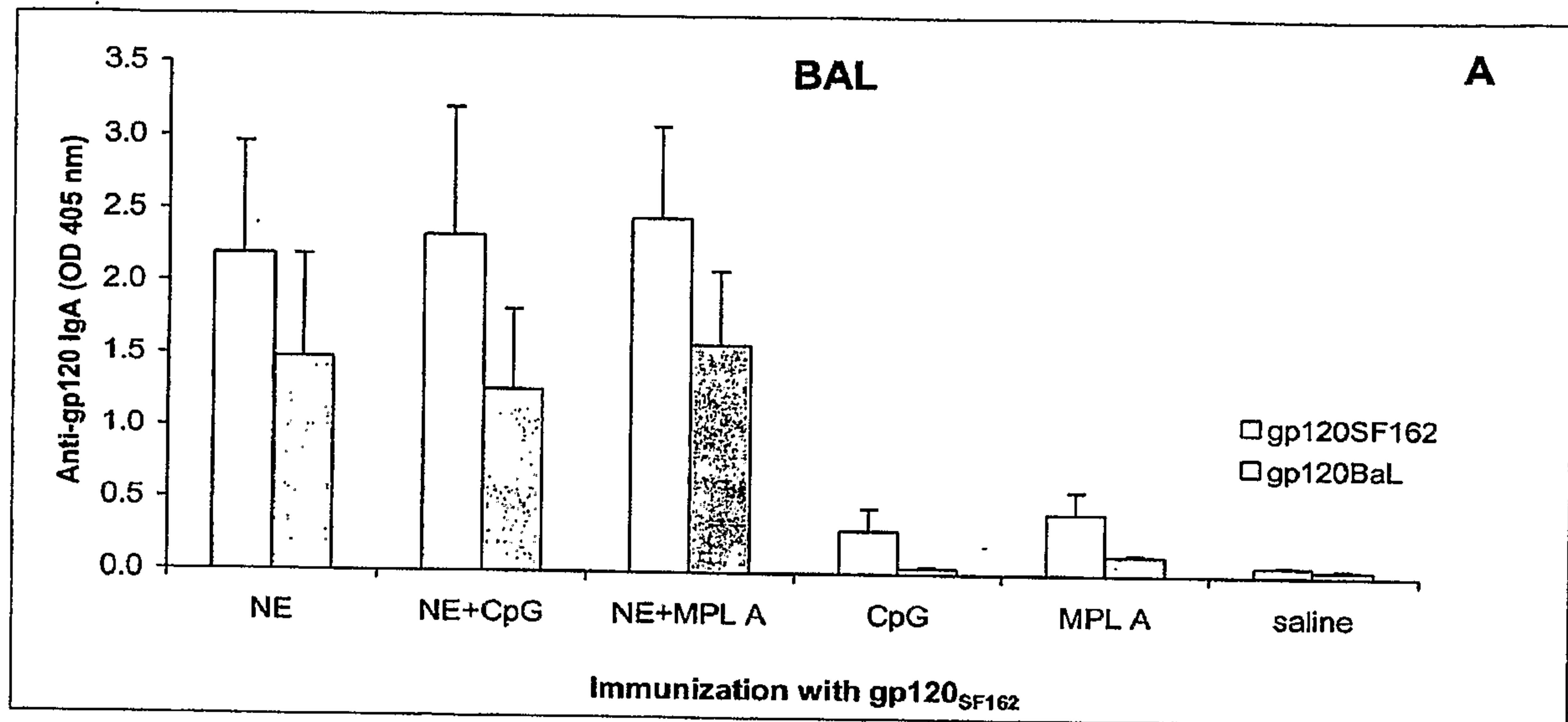


FIGURE 15B

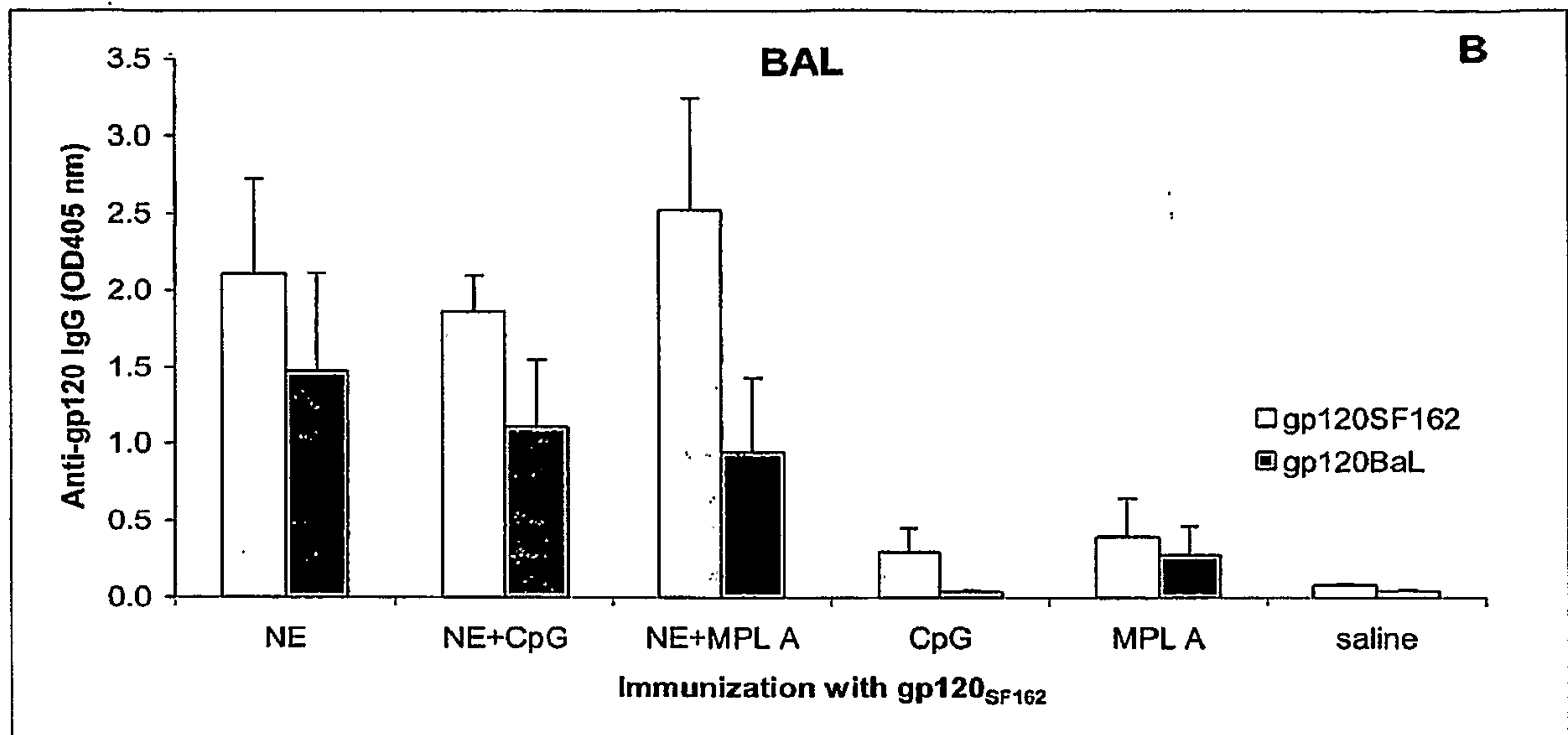


FIGURE 15C

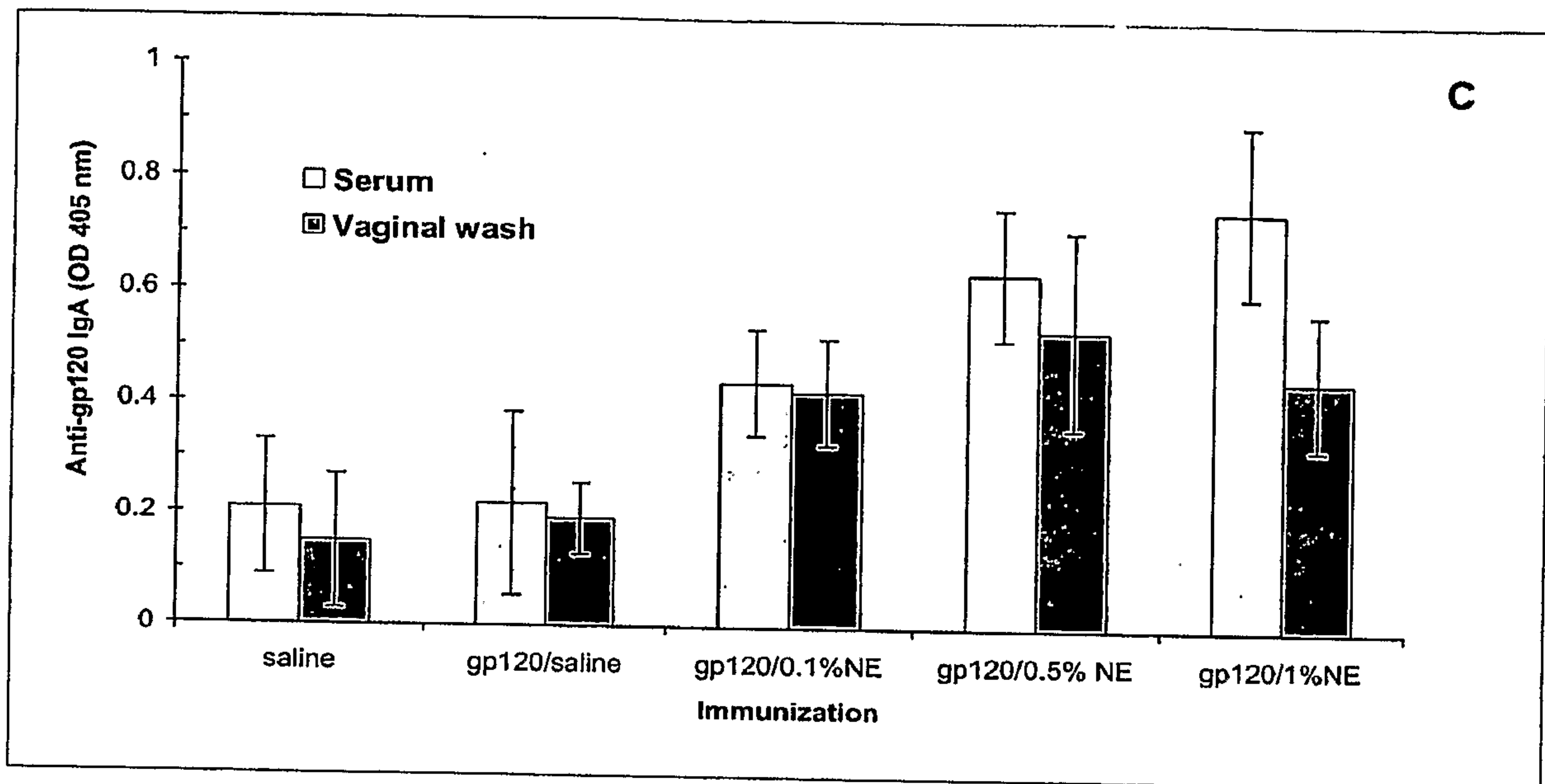


FIGURE 16A

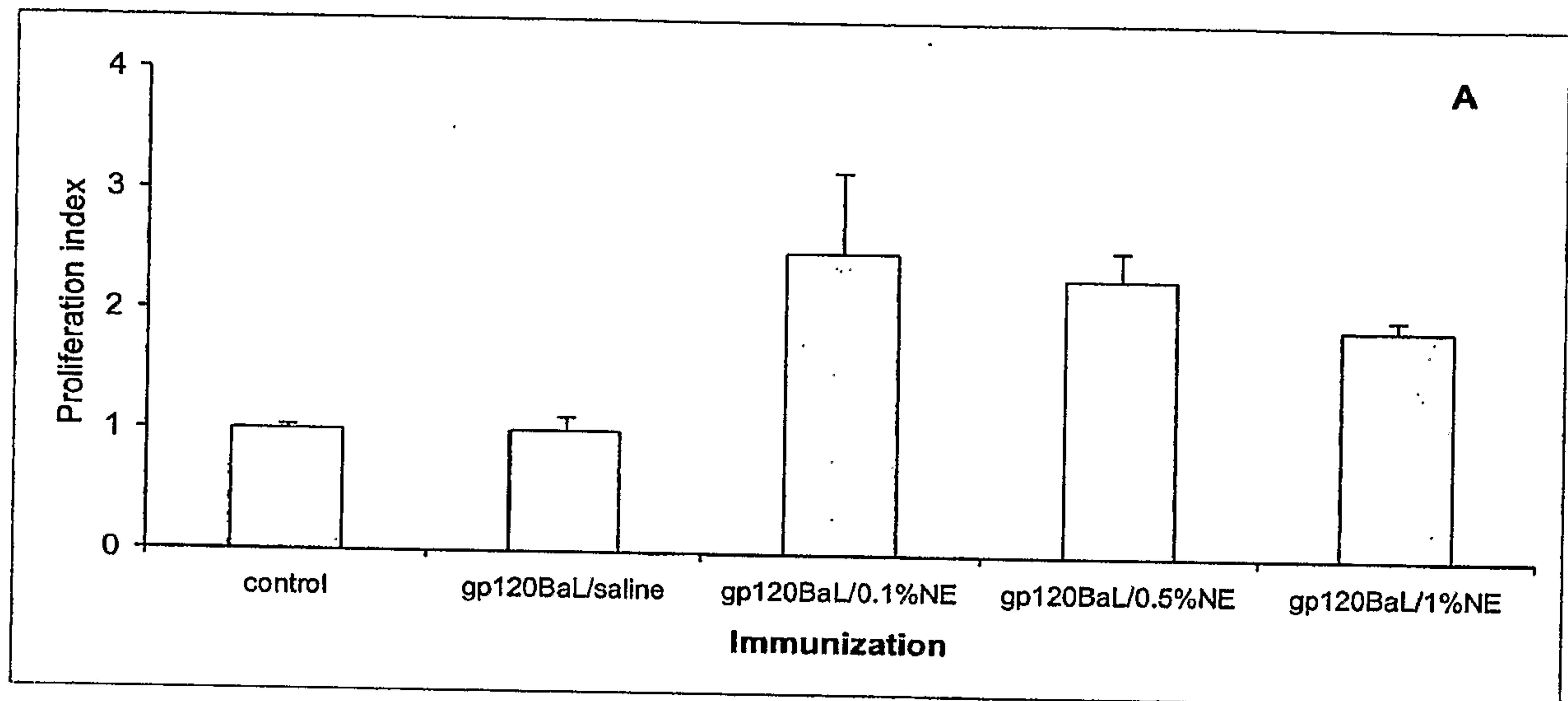


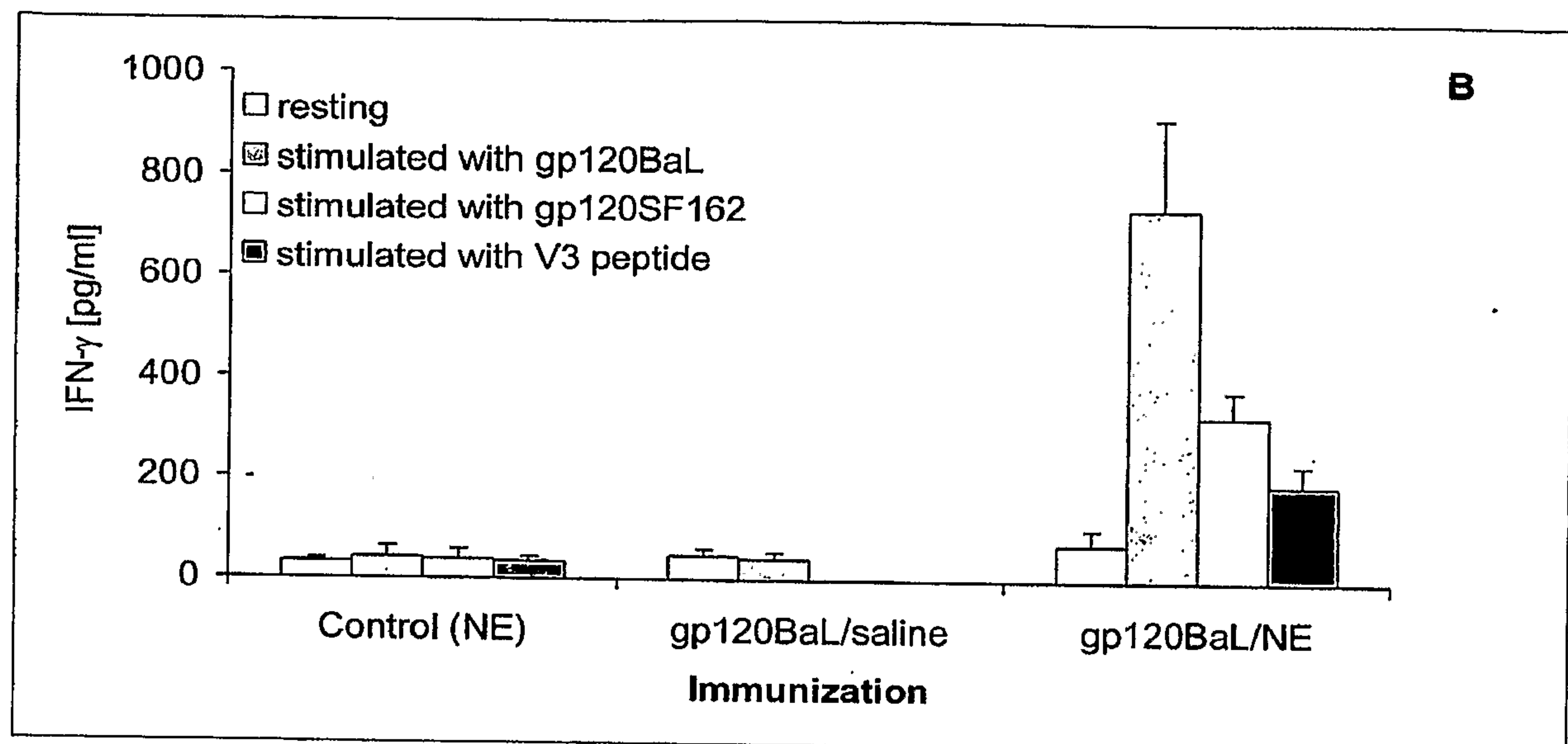
FIGURE 16B

FIGURE 17A

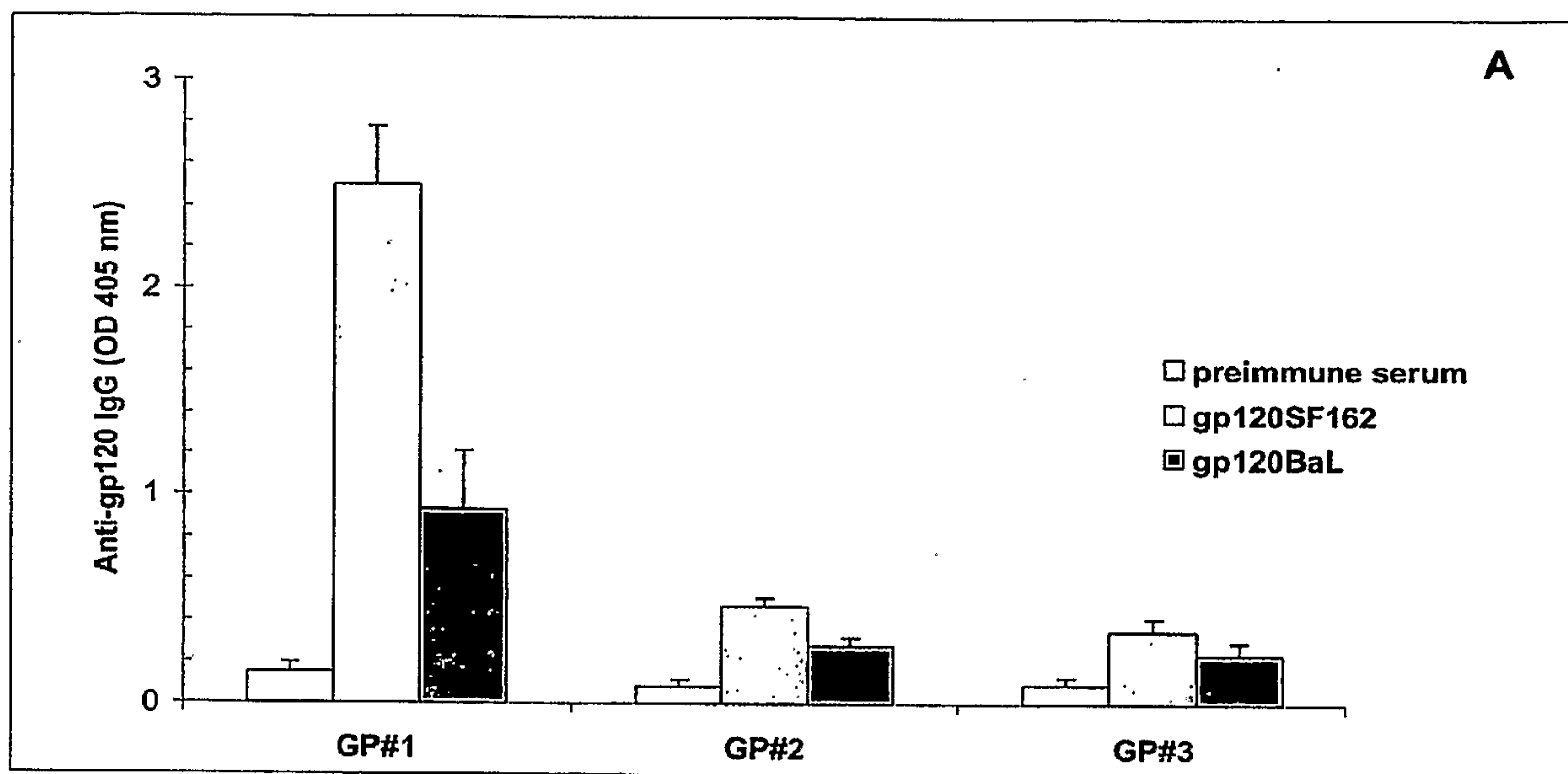


FIGURE 17B

