Mucosal Immunization with a Novel Nanoemulsion-Based Recombinant Anthrax Protective Antigen Vaccine Protects against *Bacillus anthracis* Spore Challenge[▽]

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The currently available commercial human anthrax vaccine requires multiple injections for efficacy and has side effects due to its alum adjuvant. These factors limit its utility when immunizing exposed populations in emergent situations. We evaluated a novel mucosal adjuvant that consists of a nontoxic, water-in-oil nanoemulsion (NE). This material does not contain a proinflammatory component but penetrates mucosal surfaces to load antigens into dendritic cells. Mice and guinea pigs were intranasally immunized with recombinant *Bacillus anthracis* protective antigen (rPA) mixed in NE as an adjuvant. rPA-NE immunization was effective in inducing both serum anti-PA immunoglobulin G (IgG) and bronchial anti-PA IgA and IgG antibodies after either one or two mucosal administrations. Serum anti-PA IgG2a and IgG2b antibodies and PA-specific cytokine induction after immunization indicate a Th1-polarized immune response. rPA-NE immunization also produced high titers of lethal-toxin-neutralizing serum antibodies in both mice and guinea pigs. Guinea pigs nasally immunized with rPA-NE vaccine were protected against an intradermal challenge with ~1,000 times the 50% lethal dose (~1,000 × LD₅₀) of *B. anthracis* Ames strain spores (1.38 × 10³ spores), which killed control animals within 96 h. Nasal immunization also resulted in 70% and 40% survival rates against intranasal challenge with 10 × LD₅₀ and 100 × LD₅₀ (1.2 × 10⁶ and 1.2 × 10⁷) Ames strain spores. Our results indicate that NE can effectively adjuvant rPA for intranasal immunization. This potentially could lead to a needle-free anthrax vaccine requiring fewer doses and having fewer side effects than the currently available human vaccine.

Until recently, new vaccines for inhalational anthrax were not aggressively pursued because anthrax was regarded as a rare infection with an effective vaccine. The currently licensed United Kingdom and U.S. human anthrax vaccines were developed over 30 years ago and consist of supernatants from toxigenic strains of *Bacillus anthracis* cultures adsorbed on alum (aluminum potassium sulfate) or Alhydrogel (aluminum hydroxide) (8, 47). To develop and maintain protective immunity in humans, these vaccines must be administered subcutaneously six times over 18 months, and they require yearly booster injections (7, 40). The current vaccines are also associated with local side effects from the alum adjuvant and have shown only partial protection from infection with some strains of *B. anthracis* in animal models (10, 39). After the intentional release of anthrax spores in 2001, it was clear that a more effective, easily administered, and safer vaccine was needed for emergency situations (2, 14, 25, 41).

B. anthracis secretes a tripartite toxin comprised of a protective antigen (PA) (83 kDa), a lethal factor (LF) (90 kDa), and an edema factor (89 kDa) (1, 23, 32). PA, a cell receptor-binding protein, is considered a primary immunogen for the development of protective immunity against anthrax (20, 24,

25). Immunity to PA has been shown to protect animals against inhalational anthrax (21, 55). Recent research has focused on the design of a recombinant PA (rPA) vaccine which would eliminate the need for filtered culture supernatants or whole *B. anthracis* lysate, as well as produce a more consistent immunogen (44, 53).

While most work with rPA has focused on intramuscular vaccination with alum, a vaccine applied to mucosal surfaces without the need for injection would be preferable for the rapid immunization of large, at-risk populations after potential exposure to anthrax. Also, mucosal immunization leads to both mucosal and systemic immunity (9, 31), which may be of value in preventing inhalation anthrax. Mucosal vaccine development has been limited mainly due to the lack of effective mucosal adjuvants. While several new human adjuvants have been studied, including monophosphorylated lipid A (MPL A), saponin QS-21, and muramyl tripeptide linked with dipalmitol phosphatidylethanolamine, these have been investigated predominantly for injectable vaccines (5, 20, 34). Recent attempts at mucosal vaccines for rPA involve adjuvants using soy phosphatidyl choline, cholera toxin (CT), and CpG oligonucleotides (6, 13). However, the development of Bell's palsy, associated with a nasal influenza vaccine adjuvanted with a bacterial toxin, raises safety concerns about the use of inflammatory materials as mucosal adjuvants (35).

This study examines the use of soybean oil-and-water nanoemulsions (NEs) (NanoBio Corporation, Ann Arbor, MI) as a mucosal adjuvant for an rPA vaccine. We have previously demonstrated that these NEs have broad antimicrobial activity

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(3, 17) and are safe and effective noninflammatory mucosal adjuvants for a whole-virus-based influenza vaccine (36). NEs in the present studies are simply mixed with rPA and applied to the nares of mice and guinea pigs for characterization of anti-PA immune responses. We assessed the induction of both mucosal and systemic anti-PA antibodies by immunization with these formulations, evaluated the ability of the animals' sera to neutralize anthrax lethal toxin (LeTx), and tested for protective immunity with *B. anthracis* Ames strain spore challenges. Our results show that the NE is potentially an effective adjuvant for an rPA mucosal vaccine.

MATERIALS AND METHODS

Animals. Pathogen-free, female BALB/c and CBA/J mice (5 to 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 at the University of Michigan, the Institutional Animal Care and Use Committee at the University of Texas Medical Branch at Galveston, and standard operating procedures at Battelle Memorial Institute, Columbus, OH.

Reagents. *B. anthracis* rPA and 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') (33), containing nonmethylated CpG repeats, was synthesized by Integrated DNA Technologies (Coralville, IA). The *Escherichia coli* MPL A (catalog no. L-6638), phytohemagglutinin phosphate (PHA-P), bovine serum albumin (BSA), dithiothreitol, and other chemicals used in buffers were purchased from Sigma-Aldrich Corporation (St. Louis, MO). The phosphate-buffered saline (PBS) and cell culture media were purchased from GIBCO (Grand Island, NY), and fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). The alkaline phosphatase (AP)-conjugated antibodies, goat anti-mouse immunoglobulin G (IgG) (catalog no. A-3562), and goat anti-mouse IgA (α -chain specific; catalog no. A-4937) were purchased from Sigma, and goat anti-mouse IgE horseradish peroxidase (HRP) conjugate was purchased from Bethyl, Montgomery, TX (catalog no. A90-115P). The cell proliferation kit (XTT) was purchased from Roche Diagnostics.

rPA-adjuvant formulations. NE (formulation W₂₀SEC) was supplied by NanoBio Corporation, Ann Arbor, MI. This NE is manufactured by the emulsification of cetyl pyridium chloride (1%), Tween 20 (5%), and ethanol (8%) in water with hot-pressed soybean oil (64%), using a high-speed emulsifier. Other than the cetyl pyridium chloride, W₂₀SEC is formulated with surfactants and food substances considered "generally recognized as safe" by the FDA. W₂₀SEC can be manufactured under good manufacturing practices and is stable for at least 18 months at 40°C without any special storage conditions. NE diameter was determined by dynamic light scattering using the NICOMP 380 ZLS (PSS NICOMP Particle Sizing Systems, Santa Barbara, CA). The mean droplet size was consistently below 400 nm.

All rPA-NE formulations were prepared 30 to 60 min prior to immunization by mixing rPA protein solution with NE, using saline as a diluent. Mouse immunization studies were performed using a 20- μg dose of rPA mixed with NE concentrations of 0.1% 0.5%, 1%, and 2%. For immunization with immunostimulants, 20 μg rPA was mixed with either 5 μg of MPL A or 10 μg CpG oligonucleotides in saline. The rPA aluminum hydroxide formulation (rPA-Alu) (catalog no. A-8222; Sigma) was prepared following the adsorption procedure described by Little et al. (27). Guinea pig immunization studies were performed with 10- μg , 50- μg , and 100- μg doses of rPA mixed with 1% NE and saline as a diluent. The immunization volume was 10 μl /nare for mice and 50 μl /nare for guinea pigs.

Immunization procedures. For each experiment, groups of mice ($n = 5$) were immunized intranasally 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 and 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 with one or two administra-

tions 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.

Collection of blood, bronchial alveolar lavage (BAL) fluid, 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, preterminal mice. Serum was obtained from blood by centrifugation at $1,500 \times g$ for 5 min after allowing it to coagulate for 30 to 60 min 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 22-gauge 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 dithiothreitol 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 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 (2×10^6 to $4 \times 10^6/\text{ml}$) 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 enzyme-linked immunosorbent assay (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 min 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- μl /well aliquots were incubated in rPA-coated plates for 1 h at 37°C . Plates were washed three times with PBS-0.05% Tween 20, followed by 1 h of incubation with either anti-mouse IgG or anti-mouse IgA AP-conjugated antibodies (Rockland), and then were washed three times and incubated with the AP substrate Sigma Fast (Sigma). The colorimetric reaction was stopped with 1 N NaOH according to the manufacturer's protocol, and readouts were performed using a Spectra Max 340 ELISA reader (Molecular Devices, Sunnyvale, CA) at 405 nm and the reference wavelength of 690 nm. The end point titers were recorded, and in the case of BAL fluid, the final antibody concentrations were calculated as described by Rhie et al. (43) 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, and were 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 AP conjugate was used for detection (Rockland). Antibody concentrations are presented as the mean $\pm$ standard error of the mean (SEM) of end point titers.

Dot blot detection of IgE. Saline rPA solution (2 μl ; 5 $\mu\text{g}/\text{ml}$) was adsorbed onto Nytran membranes (0.2- μm pore; Schleicher and Schuell, Keene, NH) and air dried for 30 min at room temperature. The membrane was blocked with PBS with 1% dry milk for 30 min and then washed three 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. Duplicate samples (2 μl) of each dilution were placed over the antigen spots and incubated at room temperature for 30 min. Following three washes in PBS, the dot blot was incubated with a 1:1,000 dilution of anti-mouse IgE HRP-conjugated antibody. After five washes with PBS, the dot blot was incubated with HRP substrate until dots were visible.

LeTx cytotoxicity and neutralizing antibody assay. Neutralizing antibody assay was performed using serial dilutions of sera incubated for 1 h with LeTx (consisting of 0.1 $\mu\text{g}/\text{ml}$ rPA and 0.1 $\mu\text{g}/\text{ml}$ rLF in PBS). The antibody-toxin mixtures were then added to RAW264.7 cells (20,000 to 30,000 cells/well) and incubated for 4 to 6 h at 37°C . Cell viability was assessed by the XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyanilide) assay. The serum titers resulting in 50% protection against LeTx cytotoxicity (50% neutralizing concentration [NC₅₀]) were calculated from the cell viability curves and presented as the mean value for the individual sera. Samples were assayed in triplicate at least two different times.

Live spore challenge. Challenge experiments were performed at the biosafety level 4 and 3 facilities at Battelle Memorial Institute (Columbus, OH) and the University of Texas Medical Branch (Galveston, TX), respectively. The intradermal challenges were performed according to Battelle study no. 556-G607602. Briefly, *B. anthracis* (Ames strain) spores were enumerated and diluted for intradermal spore challenge. A concentrated stock solution of Ames Battelle lot B22 was diluted in sterile water to an anticipated concentration of 5×10^3

FIG. 1. Time course of serum anti-PA IgG in mice. Mice were intranasally immunized with two doses of vaccine (arrows). (A) Induction of anti-PA IgG in CBA/J mice vaccinated with 20 μ g rPA and increasing concentrations of NE. Inset, 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 end point titers ($n = 5$ mice per group). *, statistical difference between the titer achieved with rPA-NE vaccination and the antibody titers in the other groups ($P < 0.05$). Inset, presence of anti-PA IgE in mice immunized with rPA-Alu, showing immuno-dot blots of 1:10 to 1:80 dilutions of pooled sera from mice immunized with rPA-NE (row 1), rPA-MPL A (row 2), rPA-CpG (row 3), control (row 4), and rPA-Alu (row 5).

CFU/ml. On study day 0, guinea pigs were intradermally challenged with a target dose of ~ 500 spores (0.1 ml). Postchallenge enumeration of spores revealed the actual number to be 1,380, which corresponds to 1,000 times the intradermal 50% lethal dose (intradermal $1,000 \times \text{LD}_{50}$). 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 postchallenge. Intranasal 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 and magnesium for intranasal 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 the intranasal $10 \times \text{LD}_{50}$ and $100 \times \text{LD}_{50}$. Postchallenge observation of guinea pigs was performed as described above for intradermal challenge.

Proliferation assay. The proliferation of mouse splenocytes was measured by assay of 5-bromo-2-deoxyuridine incorporation, using cell proliferation ELISA (Roche Molecular Biochemicals, Mannheim, Germany). In brief, the cells were incubated in the presence of rPA (5 μ g/ml) or PHA-P mitogen (2 μ g/ml) for 48 h and then pulsed with 5-bromo-2-deoxyuridine for 24 h. 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 cytokine expression in vitro. Freshly isolated mouse splenocytes were seeded at 2×10^6 cells/0.5 ml (RPMI 1640, 2% FBS) and incubated with rPA (5 μ g/ml) or PHA-P mitogen (2 μ g/ml) for 72 h. Cell culture supernatants were harvested and analyzed for the presence of cytokines. Interleukin-2 (IL-2), IL-4, gamma interferon (IFN- γ), and tumor necrosis factor alpha (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 $\pm$ SEM. Statistical significance was determined by analysis of variance using the Student *t* and Fisher exact tests. All tests were at 95% confidence (two tailed). A *P* value of < 0.05 was considered to be statistically significant.

RESULTS

Physicochemical analysis of rPA-NE vaccine formulations used in this study indicated that NE stabilized rPA protein, which retained a nonaltered structure and was protected

FIG. 2. Anti-PA IgA and IgG antibodies in BAL fluid. The anti-PA IgA (A) and anti-PA IgG (B) levels determined by ELISA of BAL fluid from BALB/c mice intranasally vaccinated with various formulations of vaccine are shown. Anti-PA IgA and anti-PA IgG antibodies are expressed as the mean $\pm$ SEM of antibody concentrations ($n = 5$). *, statistical difference between rPA-NE vaccination and other immunization groups ($P < 0.05$).

against progressive degradation due to deamidation observed in a buffer solution (15, 57). In addition, the stability, appearance, and size (359 ± 109 nm) of NE adjuvant were not altered by the addition of the rPA protein.

rPA-NE immunization induces serum anti-PA antibodies.

The effect of the NE 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% NE. A rapid induction of anti-PA antibodies in serum was obtained in all vaccinated animals, with some dependence on the concentration of the NE. All CBA/J mice developed high titers of serum anti-PA IgG (end point titers ranging from 10^4 to 10^5) at 5 weeks, after only two administrations of the vaccine (at 1 day and at 3 weeks). Further assays at 8 to 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 among animals intranasally immunized with rPA in saline (Fig. 1A). The pattern of the IgG subtype antibodies indicated a prevalence of IgG2a and IgG2b over IgG1, thus suggesting Th1 polarization of the immune response (Fig. 1A, inset). To further characterize the immune response generated by intranasal NE, BALB/c mice were immunized with 20 μ g rPA mixed with 1% NE (rPA-NE) and compared to mice immunized with 20 μ g rPA mixed with either MPL A (rPA-MPL A), unmethylated CpG ODN (rPA-CpG), or aluminum hydroxide (rPA-Alu) (39, 40, 42, 43). After two administrations of each formulation, all mice immunized with rPA-NE were

seropositive, with anti-PA IgG end point titers of at least 10^5 , compared to titers ranging from 10^2 to 10^3 in the rPA-MPL A, rPA-CpG, and rPA-Alu immunization groups (Fig. 1B). Again, 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, which revealed anti-PA IgE (detectable at least a 1:80 dilution in dot blots) in mice intramuscularly immunized with rPA-Alu but not in any other group (Fig. 1B, inset). This is consistent with prior reports of alum adjuvant-based vaccines inducing a Th2 response (22, 26).

Intranasal rPA-NE vaccination produces mucosal immunity. We hypothesized that nasal immunization may induce mucosal immunity to help protect against respiratory infection (9, 58). Significant levels of anti-PA-specific secretory IgA antibodies were observed in BAL samples from BALB/c mice vaccinated with rPA-NE (Fig. 2A). A similar pattern, with higher antibody concentrations, was detected for anti-PA IgG in BAL samples (Fig. 2B). The animals with titers of secretory IgA in BAL fluid also had detectable levels of serum anti-PA IgA. These data show that significant mucosal responses can be induced with an intranasal vaccine consisting of rPA in NE, but not with other formulations or with intramuscular immunizations (data not shown). No inflammatory response was observed in histopathological examination of animals' nasal mucosa after administration of NE with or without antigen, indicating that the NE is not proinflammatory.

The rPA-NE vaccine produces neutralizing antibodies against anthrax toxin in mice. To evaluate whether the mucosal NE-

FIG. 3. 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). Error bars indicate SEMs.

based vaccine could produce toxin-neutralizing antibodies, sera from immunized mice were tested for the ability to neutralize anthrax LeTx. Sera from mice immunized with rPA-NE were effective in neutralizing LeTx and prevented RAW264.7 cell death, with an NC₅₀ of $>10^3$. In contrast, sera from mice immunized with either rPA-MPLA, rPA-CpG, or rPA-Alu had NC₅₀s of <10 . Naive control sera or sera from mice immunized with rPA in saline did not inhibit LeTx cytotoxicity at any concentration (Fig. 3).

rPA-NE immunization yields Th1 cellular responses. PA antigen-specific cellular responses were measured in a proliferation assay (Fig. 4) and through the analysis of the cytokine secretion from splenocytes stimulated in vitro with rPA (Table 1). As shown in Fig. 4, rPA stimulated proliferation only 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 from mice vaccinated with rPA-NE showed increased expression of IFN- γ , TNF- α , and IL-2 but failed to produce IL-4, compared to the control (nonstimulated) cells. This suggests that nasal immunization with rPA-NE yields antigen-specific Th1-type polarization of cellular responses (Table 1).

rPA-NE vaccines protect guinea pigs against intradermal live spore challenge. Three groups of three 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 end point antibody titers of

FIG. 4. PA-specific induction of splenocyte proliferation in vitro. Splenocytes isolated from immunized mice were stimulated with rPA (5 μ g/ml) for 72 h. Proliferation indexes were calculated as a ratio of the activity in rPA-stimulated cells to the activity in resting splenocytes. *, statistical difference between groups ($P < 0.05$). Error bars indicate SEMs.

TABLE 1. Antigen-specific cytokine expression in splenic lymphocytes from BALB/c mice immunized with rPA-NE^a

Cytokine	Mean concn (pg/ml) $\pm$ SEM ^b in splenic lymphocytes from:			
	rPA-NE-immunized mice		Control mice	
	-PA	+PA	-PA	+PA
IFN- γ	26.37 $\pm$ 8.10	93.30 $\pm$ 24.40*	13.23 $\pm$ 2.33	15.40 $\pm$ 3.70
TNF- α	4.74 $\pm$ 2.62	22.62 $\pm$ 12.85*	3.51 $\pm$ 2.34	2.50 $\pm$ 2.50
IL-2	27.10 $\pm$ 2.62	59.00 $\pm$ 16.9*	33.00 $\pm$ 17.30	35.00 $\pm$ 16.90
IL-4	17.44 $\pm$ 2.17	21.76 $\pm$ 9.76	19.00 $\pm$ 2.73	18.17 $\pm$ 2.69

^a rPA-stimulated cytokine responses in harvested splenocytes in vitro are shown. Th1-type cytokine (IFN- γ , TNF- α , and IL-2) and Th2-type cytokine (IL-4) production was evaluated by specific ELISA of culture supernatant fluid from nonstimulated (-PA) and rPA-stimulated (+PA) cells.

^b *, statistically significant difference in cytokine concentrations between resting and rPA-stimulated cells ($P < 0.05$).

$>1 \times 10^5$. 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 (Fig. 5A). At 6 months, the animals were challenged intradermally with $1,000 \times$ LD₅₀ 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 intradermal challenge, while none of the control animals survived (Fig. 5B). An LeTx neutralization assay before the challenge documented mean serum NC₅₀ titers of 3×10^2 in the group immunized with 10 μ g rPA-NE and of $\sim 1 \times 10^3$ in the groups immunized with 50 μ g and 100 μ g rPA-NE (Fig. 5B insert).

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 ($n = 10$) were immunized with formulations containing 10, 50, and 100 μ g rPA mixed with 1% NE. Immunization consistently produced 100% seroconversion and significant anti-PA IgG responses in all immunized animals. A boost at 4 weeks resulted in the rapid increase of anti-PA IgG in the serum, producing end point antibody titers of $>1 \times 10^5$ in all groups. An LeTx neutralization assay before the challenge indicated mean NC₅₀ titers of 1×10^3 to 2×10^3 in all vaccinated groups (Fig. 6A). At 7 weeks the animals were challenged intranasally with either $10 \times$ LD₅₀ (1.2×10^6 spores) or $100 \times$ LD₅₀ (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 $10 \times$ LD₅₀ challenge and 40% after the $100 \times$ LD₅₀ challenge (Fig. 6B and C, respectively). The results of the $10 \times$ LD₅₀ challenge suggest that the mucosal rPA-NE vaccine produced protective immunity comparable to that after intramuscular vaccination using rPA with alum (37). 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 (Table 2).

FIG. 5. Immune response and survival of guinea pigs intranasally immunized with rPA-NE vaccine. Hartley guinea pigs were vaccinated with 2 doses of vaccine (at 1 day and 4 weeks, as indicated 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 end point titers $\pm$ SEM). (B) Intradermal challenge. At 6 months, guinea pigs were intradermally injected with $1,000 \times LD_{50}$ of Ames spores, and mortality was monitored for 14 days. For vaccinated and control animals ($n = 9$; inset), LeTx neutralization was performed at 22 weeks before the challenge. The antibody titer in which RAW264.7 cells retained 50% viability (NC_{50}) was determined from the cell viability obtained in at least two assays, each performed in triplicate. *, statistically significant difference compared to unvaccinated animals ($P < 0.001$).

DISCUSSION

The development of safe and efficacious adjuvants is crucial to produce new mucosal vaccines to protect against inhalation anthrax. In general, antigens delivered via the mucosa are poorly immunogenic and are easily degraded by mucosal enzymes. This has led to concerns that this approach may induce a state of tolerance instead of protective immunity (18, 46, 58). In this study, we present a novel mucosal adjuvant for anthrax vaccination that is both potent and free of toxicity. These stable and convenient preparations of NEs mixed with the rPA of *B. anthracis* have induced significant mucosal and systemic immune responses. The NE adjuvant is effective over a broad range of antigen concentrations, and only a single application (in guinea pigs) or two vaccinations (in mice) are necessary to induce protective immune responses. Despite the nasal route of immunization, significant protection against either intradermal or intranasal spore challenge was achieved.

The guinea pig is a primary model for testing anthrax vaccine efficacy (19, 20, 42). In our intranasal rPA-NE studies, complete protection was obtained against intradermal chal-

lenge with $1,000 \times LD_{50}$ of *B. anthracis* spores 5 months after booster immunization. This suggests that a durable anti-PA IgG response with neutralizing antibody titers of greater than 10^2 provides protection from dermal anthrax exposure. In comparison, intramuscular immunization with the commercial anthrax vaccine resulted in overall survival rates of 53% to 63% in guinea pigs after intramuscular challenges with $10 \times$ to $1,000 \times LD_{50}$ of Ames strain spores, and IgG titers alone did not predict protection (19). A high concentration (NC_{50} of $>10^3$) of the LeTx-neutralizing antibodies present in the sera of mice and guinea pigs after rPA-NE immunization indicates that mixing with NE adjuvant did not denature essential protective epitopes in the antigen that are important for protection against infection with live *B. anthracis* (44). Inhalational challenge of guinea pigs intranasally vaccinated with rPA-NE yielded 70% and 40% survival for $10 \times$ and $100 \times LD_{50}$ of Ames strain spores, respectively, while the neutralizing antibody titers were comparable (or higher) to these in fully protected intradermally challenged guinea pigs. These results were similar to those for other anthrax vaccines (21, 27) and suggest that anti-PA IgA, IgG, and neutralizing antibodies are

FIG. 6. Immune response and intranasal challenge of guinea pigs intranasally vaccinated with rPA-NE vaccine. Hartley guinea pigs ($n = 10$ per group) were vaccinated at 1 day and 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 end point titers. The LeTx neutralization assay cell was performed before the challenge, with values representing mean titers at which RAW264.7 cells retained 50% viability (NC₅₀). (B and C) Survival curves after intranasal challenge. At 7 weeks, guinea pigs were infected by intranasal instillation of 10 $\times$ LD₅₀ (B) and 100 $\times$ LD₅₀ (C) of Ames spores, and animals were monitored up to 16 days. P was <0.05 between all vaccinated groups compared to unvaccinated animals.

not fully predictive for protection against inhalation anthrax. In addition, the intranasal challenge results indicate a complex mechanism of protection against inhalation infection with anthrax (50). However, even when not providing complete sur-

vival, rPA-NE immunization significantly extended the TTD (approximately 3 to 5 days) after intranasal challenge. This may provide potential therapeutic advantages for when mucosal rPA-NE immunization is used for postexposure anthrax

TABLE 2. Mortality of guinea pigs after intranasal challenge with *B. anthracis* spores

Vaccination	10× LD ₅₀ challenge		100× LD ₅₀ challenge	
	Mortality	TTD ^a (mean ± SD)	Mortality	TTD (mean ± SD)
PBS control	10/10	3.8 ± 1.4	10/10	3.1 ± 1.1
NE control	ND ^b	ND	10/10	3.5 ± 1.1
10 µg rPA-NE	3/10	7.0 ± 1.4*	6/10	4.3 ± 1.2
50 µg rPA-NE	3/10	8.3 ± 1.2*	6/10	6.0 ± 1.4*
100 µg rPA-NE	3/10	9.7 ± 3.1*	6/10	8.3 ± 1.2*

^a *, significant differences ($P < 0.05$) between TTDs of vaccinated animals and controls.

^b ND, not determined.

prophylaxis in combination with antibiotics or anti-PA monoclonal antibodies (38, 52).

Although it is difficult to directly compare titers of anti-PA IgG antibodies achieved with various adjuvants and routes of administration, the NE appeared to generate immune responses at least equivalent to those with other adjuvants. CT increased the effectiveness of the rPA-based vaccine (13, 45). However, unlike for the NEs, NIAID has raised safety concerns about CT, because when delivered intranasally it can transit the cribiform plate via the olfactory nerve and can cause inflammation in the olfactory region of the brain (12, 49). In comparable studies using the same guinea pig intranasal challenge model (and performed at the same facility with the same protocol), intramuscular immunization with rPA adsorbed on Alhydrogel and immunization with the commercial AVA vaccine provided 100 and 70% protection, respectively, when challenge was with $5 \times \text{LD}_{50}$ of anthrax Ames strain spores (37). We used higher challenge doses and obtained equivalent to better protection, suggesting similar immunogenicity. Also, our intranasal vaccinations with rPA-NE vaccine typically resulted in at least 10^4 - to 10^5 -fold-higher titers of anti-PA antibodies than immunization with nonadjuvanted rPA. Therefore, NE adjuvants that both demonstrate high effectiveness and are made from “generally recognized as safe” materials and vegetable oils could be considered for use as safe and effective candidates for adjuvants in mucosal vaccines (17, 36).

Development of a protective humoral anti-PA response in humans or in various experimental animal models requires multiple immunizations with either parenteral or mucosal anthrax vaccines (6, 13, 21, 29, 30). This cumbersome administration schedule and the short duration of protective immunity are serious disadvantages for anthrax immunization in response to bioterrorist attacks. A recent study reported that two intramuscular vaccinations of macaques with rPA bound to Alhydrogel produced higher serum IgG responses than the licensed AVA vaccine. However, the IgG levels in those animals significantly decreased by 6 to 10 weeks after immunization (54). The reasons for the limited duration of immunity with these vaccines are unclear, and a direct comparison is complicated by differences in experimental models, but it is possible that the inherent instability of rPA leads to the degradation of critical antigenic epitopes (15, 16, 57). We found that the rPA-NE vaccine was effective in inducing high titers of anti-PA IgG after only two intranasal administration and produced durable, long-term (6-

month) neutralizing immunity. Incubation with NE seemed to decrease degradation of rPA, possibly stabilizing its antigenicity (data not shown). Thus, the increased stability of the antigen may play a role in the enhanced adjuvant activity observed with NE.

Most studies of anthrax vaccines focus on serum IgG concentration as the primary marker of protective immunity (2, 25, 29). However, the NE adjuvant also induced mucosal immunity to PA. Significant concentrations of anti-PA IgA and IgG antibodies in mucosal secretions (BAL fluid) were detected in mice immunized with rPA-NE, although the value of mucosal immunity in protection against inhalation anthrax is unclear. rPA administered with either CpG ODN or MPL A immunostimulatory adjuvant (4, 51) was not effective in inducing a mucosal immune response. However, inclusion of MPL A into the NE-based vaccines resulted in a more rapid induction of serum anti-PA IgG but no change in the overall titer of anti-PA antibodies (data not shown). MPL A has proved to be an effective inducer of Th1 polarization of the immune response (48). An examination of the PA-specific cytokine secretion pattern in splenocytes after rPA-NE immunization and the prevalence of IgG2a and IgG2b subtype antibody suggest that the NE-based vaccine may result in a similar Th1 polarization (6, 11, 28). In contrast, the animals immunized with rPA-Alu had elevated levels of IgE and poor IgG and IgA responses, suggesting that their cellular immunity is biased toward a Th2 type of response (56). Thus, rPA-NE yields a serum and systemic adjuvant activity similar to that with MPL A, which may be why the combination of these adjuvants did not appear to be synergistic when administered intranasally.

In summary, our study indicates that NEs appear to be an effective mucosal adjuvant for a rPA anthrax vaccine. These formulations induce long-lasting, robust, and specific humoral and cellular responses; appear to lack adverse effects; and have the ability to stabilize the antigen.

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