RESEARCH ARTICLE

Nasal immunization with the mixture of PA63, LF, and a PGA conjugate induced strong antibody responses against all three antigens

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Abstract
A new generation anthrax vaccine is expected to target not only the anthrax protective antigen (PA) protein, but also other virulent factors of Bacillus anthracis. It is also expected to be amenable for rapid mass immunization of a large number of people. This study aimed to address these needs by designing a prototypic triantigen nasal anthrax vaccine candidate that contained a truncated PA (rPA63), the anthrax lethal factor (LF), and the capsular poly-\(\gamma\)-d-glutamic acid (\(\gamma\)DPGA) as the antigens and a synthetic double-stranded RNA (dsRNA), polyriboinosinic-polyribocytidylic acid (poly(I:C)) as the adjuvant. This study identified the optimal dose of nasal poly(I:C) in mice, demonstrated that nasal immunization of mice with the LF was capable of inducing functional anti-LF antibodies (Abs), and showed that nasal immunization of mice with the prototypic triantigen vaccine candidate induced strong immune responses against all three antigens. The immune responses protected macrophages against an anthrax lethal toxin challenge in vitro and enabled the immunized mice to survive a lethal dose of anthrax lethal toxin challenge in vivo. The anti-PGA Abs were shown to have complement-mediated bacteriolytic activity. After further optimization, this triantigen nasal vaccine candidate is expected to become one of the newer generation anthrax vaccines.

Introduction

Bacillus anthracis, the etiological agent of anthrax, is a rod-shaped, nonmotile, Gram-positive bacterium that forms highly resistant endospores under adverse conditions (Ascenzi et al., 2002). Depending on the site of inoculation, anthrax infection can occur in three clinical forms: cutaneous, gastrointestinal, or inhalational. Cutaneous and gastrointestinal anthrax are rare and highly treatable, but inhalation anthrax has a fatality rate approaching 100%, with death occurring shortly after the onset of symptoms (Friedlander, 1999). Upon inhalation, anthrax spores are deposited into the alveolar spaces of the respiratory tract of the host and ingested as inert particles by local macrophages (Dixon et al., 1999). Wherein they are transported to the mediastinal and peribronchial lymph nodes, germinate into vegetative bacilli, and are released into the systemic circulation (Koehler et al., 1994). Released bacilli begin unimpeded extracellular multiplication while secreting fatal levels of anthrax toxins (Wang & Roehrl, 2005).

Vaccination is considered the best option for the prophylaxis of anthrax. Currently, the only anthrax vaccine available in the United States is the anthrax-vaccine adsorbed (AVA, now BioThrax<sup>®</sup>), an aluminum hydroxide (Alum)-adsorbed, formalin-treated culture supernatant of a toxigenic B. anthracis strain (Puziss et al., 1963). However, there still is room to improve the AVA. For example, the AVA has a lengthy and complicated dosing schedule with 6 initial injections in the first 18 months and subsequent yearly booster injections. Moreover, the real composition of the AVA is unknown, although the primary antigen component was identified to be the protective antigen (PA) protein of the anthrax toxins (Puziss et al., 1963), which prompted the development of new generation anthrax vaccines based on recombinant PA. Finally, the AVA is administered by subcutaneous needle-syringe injection, which may not be amenable for self-administration or for administration by untrained personnel, and thus, not suitable for rapid mass immunization in the case of an emergency. A newer generation anthrax vaccine is expected to address all these issues.
Bacillus anthracis has two primary virulence factors, anthrax toxins and the capsule. Anthrax toxins have three components, the PA, the lethal factor (LF), and the edema factor (EF). Individually, these components are nontoxic. However, they can combine to form two binary toxins, the lethal toxin (LeTx, PA and LF) and the edema toxin (PA and EF). The binding of the PA to receptors on the surface of host cells allows the entry of the LF and EF into the cell cytosol, where they are toxic (Bradley et al., 2001; Scobie et al., 2003; Scobie & Young, 2005). Thus, anti-PA antibodies (Abs) were shown to block the transport of LF and EF into cells and aid in the prevention of the infection. The PGA capsule is another virulence factor, by which the B. anthracis is surrounded and protected from the host immune system (Welkos et al., 1993). Bacillus anthracis strains that are encapsulated within the PGA grow unhindered in infected hosts, whereas bacteria lacking the capsule are nearly avirulent (Welkos, 1991; Welkos et al., 1993). Thus, a vaccine capable of inducing anticapsule Abs is expected to inhibit the bacillus growth, and thus, reduce the anthrax toxin production, which is expected to be beneficial. The feasibility of inducing Abs against PGA and the efficacy of the anti-PGA Abs in protecting against anthrax infection have been confirmed in several recent studies (Rhie et al., 2003; Schneerson et al., 2003; Chabot et al., 2004; Kozel et al., 2004; Joyce et al., 2006). Moreover, it was reported that after naturally occurring human infections, 68–93% of the patients developed anti-PA Abs, 43–55% developed anti-LF Abs, and 67–94% developed anti-PGA Abs (Wang & Roehrl, 2005). Furthermore, there were data showing that vaccination of guinea pigs with PA and LF together afforded a better protection against an aerosolized anthrax spore challenge than with PA alone (Ivins & Welkos, 1988). Taking all these findings together, this study aimed to develop an improved prototypic triantigen vaccine candidate that contains purified PA(63), LF, and PGA to target all three components.

To address the need for an immunization approach suitable for rapid mass immunization, this vaccine candidate was dosed intranasally. Intranasal dosing with specialized devices is expected to be amenable for self-administration or for administration by nonprofessionals.

In the present study, the immune responses induced by a nasal vaccine candidate containing PA (PA83 or PA63), LF, and a PGA conjugate were evaluated. The PA63 was used to reduce the potential toxicity when PA and LF were dosed together. It was previously shown that the PA63 fragment generated by trypsinizing PA83 was less toxic than the PA83 (Novak et al., 1992). There also were data showing that Abs against a PA63 preparation whose toxicity was up to 100-fold less than that of the PA83 protect rabbits and nonhuman primates against a lethal spore challenge (Hepler et al., 2006). The PGA was conjugated to a carrier protein to improve its immunogenicity because the PGA alone is weakly or nonimmunogenic (Rhie et al., 2003; Schneerson et al., 2003; Chabot et al., 2004). A synthetic double-stranded RNA (dsRNA), poly(I:C), was used as a vaccine adjuvant. Data from recent studies have shown that the poly(I:C) was a safe and potent nasal vaccine adjuvant (Ichinohe et al., 2005; Partidos et al., 2005; Asahi-Ozaki et al., 2006; Sloat & Cui, 2006a, b). This study showed that strong and functional immune responses against all three antigens were induced after only 2 or 3 intranasal doses with this prototypic triantigen vaccine candidate, and that the anti-PA Abs induced were as strong as that induced by s.c. injection of rPA83 adsorbed onto aluminum hydroxide gel (Alum).

Materials and methods

Bacterial strain, inoculation, and culture methods

Bacillus licheniformis was obtained from the Bacillus Genetic Stock Center (BGSC) at the Ohio State University (Columbus, OH). Highly mucoid colonies were selected and grown aerobically in Erlenmeyer flasks with E-medium (Perez-Camero et al., 1999). The formula of the E-medium in g L⁻¹ was as follows: glycerol, 80.0; citric acid, 12.0; L-glutamic acid, 20.0; NH₄Cl, 7.0; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.5; CaCl₂·2H₂O, 0.15; MnSO₄·H₂O, 0.104; FeCl₃·6H₂O, 0.04. The aforementioned chemicals were all purchased from Sigma-Aldrich (St Louis, MO). The E-medium was brought to boiling temperature, followed by shaking to dissolve all components, allowed to cool to room temperature, and adjusted to a pH of 7.4 with NaOH before autoclaving. Cultures were maintained in a shaker-incubator at 37 °C for 36–48 h.

Purification of γDPGA

The γDPGA was purified from B. licheniformis as previously described (Sloat & Cui, 2006a). The bacterial culture was centrifuged at 4 °C, 6500 g for 30 min. The supernatant was collected, and the γDPGA was precipitated with 3 volumes of ethanol at 4 °C overnight. The γDPGA precipitate was collected and dialyzed against deionized water, which was replaced every 2 h over 6–8 h. The γDPGA solution was acidified to a pH of 1.5 with HCl, followed by precipitation with three volumes of 1-propanol at −20 °C. The resultant precipitate was collected by centrifugation and washed twice with acetone and once with ethyl ether. The purified γDPGA was dissolved in water, dialyzed extensively, and lyophilized. The structure and purity of the γDPGA was verified by ¹H NMR spectroscopy (Bruker am 400 MHz FT NMR, Blue Lion Biotech, Snoqualmie, WA) and UV–Vis scanning from 190 to 300 nm (DU-640 Spectrophotometer, Beckman Coulter, Fullerton, CA), and the purity was estimated to be...
greater than 96%. The percent of the D-isomer glutamic acids can be increased to 85%, depending on the concentration of the Mn in the culture medium. Before further use, the γDPGA was sonicated for 1.5 h in an ice-cold water bath sonicator (Ultrasonic Cleaner Model 150 T, VWR International, West Chester, PA).

Synthesis of PGA–BSA conjugates

The γDPGA–BSA conjugate was prepared as previously described (Rhie et al., 2003; Sloat & Cui, 2006a). Five milligrams of bovine serum albumin (BSA, Sigma-Aldrich, catalog # A8303), 15 mg of γDPGA, and 50 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma-Aldrich) were dissolved in phosphate-buffered saline (PBS; pH 7.4, 10 mM) and stirred for 4 h at room temperature. The reaction product was desalted using a PD-10 column (GE Amersham Biosciences, Piscataway, NJ). The γDPGA–BSA conjugate was lyophilized and stored at −20°C until further use.

Nasal immunization

All mouse studies were carried out following NIH guidelines for animal use and care. For nasal dosing, BALB/c mice (female, 6–8 weeks, Simonsen Laboratories Inc., Gilroy, CA) were lightly anesthetized intraperitoneally with pentobarbital (6 mg per 100 g, Abbott Laboratories, North Chicago, IL) before dosing. To identify the optimal dose of poly(I:C), mice were nasally dosed with rPA83 (5 μg mouse−1, List Biological Laboratories Inc., Campbell, CA) admixed with various concentrations of poly(I:C) (0.1, 1.0, 10.0, 20.0, or 40.0 μg mouse−1, GE Healthcare Biosciences, Piscataway, NJ). As a negative control, mice were left untreated. Mice were dosed on days 0 and 14, euthanized on day 28, and bled by cardiac puncture.

To evaluate the feasibility of inducing functional anti-LF Abs by nasal LF, BALB/c mice were nasally dosed with various amounts of LF (5, 10, 20, or 40 μg mouse−1, BEI Resources, Manassas, VA) admixed with poly(I:C) (10 μg mouse−1). As controls, mice were either left untreated or nasally dosed with LF (5 μg mouse−1) admixed with CT (1 μg mouse−1). Mice were dosed on days 0 and 14, euthanized on day 28, and bled by cardiac puncture.

In another animal experiment, mice were nasally dosed with the mixture of rPA83 (5 μg mouse−1), LF (5 μg mouse−1), and the γDPGA–BSA conjugate (20 μg mouse−1) admixed with poly(I:C) (10 μg mouse−1). Each dose was administered in two 10 μL doses with 10–15 min between each dose, half in each nare. As a positive control, mice were also nasally dosed with the mixture of rPA83, LF, and γDPGA–BSA, adjuvanted with cholera toxin (CT, 1 μg mouse−1, List biological Labs). Negative control mice were left untreated. Mice were dosed on days 0 and 14, euthanized on day 55, and bled by cardiac puncture. Their spleens were also harvested for splenocyte proliferation assay.

Finally, BALB/c mice were nasally dosed with 20 μL of the mixture of rPA63 (15 μg mouse−1, List Biological), LF (20 μg mouse−1), and the γDPGA–BSA conjugate (20 μg mouse−1), admixed with poly(I:C) (10 μg mouse−1). As controls, mice were left untreated, s.c. injected with the mixture of rPA63, LF, and γDPGA–BSA adsorbed onto alum (50 μg mouse−1, USP grade, Spectrum), or nasally dosed with rPA63 alone (15 μg or 24 nmol) admixed with poly(I:C), rPA83 alone (20 μg or 24 nmol) admixed with poly(I:C), or the LF alone (20 μg) admixed with poly(I:C). Mice were dosed on days 0, 14, 28, and bled via the tail vein on day 42.

Quantification of Abs against PA, γDPGA, LF, and poly(I:C)

The levels of anti-PA83, anti-LF, and anti-γDPGA in the serum samples were determined using enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates were coated with 100 ng of rPA83, LF, or the pure PGA dissolved in 100 μL carbonate buffer (pH 9.6) at 4°C overnight. For anti-PA83 and anti-LF Ab measurements, the plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20) and blocked with 4% (w/v) BSA in PBS/Tween 20 for 1 h at 37°C. Samples were diluted two fold serially (or as indicated) in 4% BSA/PBS/Tween 20, added to the plates following the removal of the blocking solution, and incubated for an additional 3 h at 37°C. The samples were removed, and the plates were washed 5 times with PBS/Tween 20. HRP-labeled goat antimouse immunoglobulin (IgG, IgG1, IgG2a, IgM, or IgA, 5000-fold dilution in 1% BSA/PBS/Tween 20, Southern Biotechnology Associates Inc., Birmingham, AL) was added into the plates, followed by another hour of incubation at 37°C. Plates were again washed five times with PBS/Tween 20. The presence of bound Ab was detected following a 30 min incubation at room temperature in the presence of 3,3′,5,5′-tetramethylbenzidine solution (TMB, Sigma-Aldrich), followed by the addition of 0.2 M sulfuric acid as the stop solution. The absorbance was read at 450 nm using a SpectraMax Plate reader (Molecular Devices Inc., Sunnyvale, CA). Ab titers were derived by comparing the OD450 nm values of the samples with the OD450 nm plus 2 × SD of the negative control mice.

The anti-γDPGA Abs were determined similarly, except that the 4% BSA in PBS/Tween 20 was replaced by 10% (v/v) horse serum (Sigma-Aldrich) in PBS/Tween 20 in the plate blocking and sample dilution steps. In addition, the secondary Ab was diluted in 2.5% (v/v) horse serum in PBS/Tween 20.
The anti-dsRNA Abs were determined similarly. The plates were coated with the poly(I:C) (100 ng well⁻¹). As a negative control, the plates were coated with carbonate buffer only. As a positive control, the serum sample was replaced by a primary anti-dsRNA monoclonal antibody (J2 IgG2a, English and Scientific Consulting Bt., Szivak, Hungary). The secondary Ab used was the HRP-labeled goat antimouse IgG2a. It was confirmed that the amount of poly(I:C) bound to the plates was correlated to the concentration of the poly(I:C) solution.

**Splenocyte proliferation assay**

Proliferative immune response was evaluated by measuring splenocyte proliferation after in vitro restimulation. Spleens from individual mice were pooled, and single cell suspensions were prepared as previously described (Cui & Mumper, 2002). Splenocytes were cultured at a density of 4 × 10⁶ cells mL⁻¹ and restimulated with rPA83 (12.5 μg mL⁻¹), LF (10 μg mL⁻¹), or pure PGA (10, 50, or 100 μg mL⁻¹) in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) for 5 days at 37 °C, 5% CO₂ (Boyaka et al., 2003; Sloat & Cui, 2006c). As a control, splenocytes were cultured without restimulation. The cell numbers were determined using an MTT kit (Sigma-Aldrich). The proliferation index was reported as the ratio of the final OD₅₇₀ nm value of cells with restimulation over that without restimulation.

**Lethal toxin neutralization activity (TNA) assay**

To evaluate the functionality of the anti-γDPGA Abs induced, a complement-mediated bactericidal assay was completed as described elsewhere with modifications (Chabot et al., 2004). Due to the biohazards associated with *B. anthracis*, *B. licheniformis* was used. It has been shown that the PGAs from *B. anthracis* and *B. licheniformis* were immunologically cross-reactive (Makino et al., 1989; Message et al., 1998). Also, Abs raised against the PGA from *B. licheniformis* were shown to bind to the PGA from *B. anthracis* with high affinity, and passive immunization with the Abs against *B. licheniformis* PGA protected mice against a lethal dose of anthrax spore challenge (Kozel et al., 2004). Briefly, *B. licheniformis* spores were resuspended in LB broth and incubated at 37 °C for 90 min without shaking. These freshly germinated vegetative bacterial cells were centrifuged for 5 min at 18,000 g, and resuspended in Gey's buffer [PBS (pH 7.4, 10 mM), 1 mM CaCl₂·2H₂O, 0.5 mM MgCl₂·6H₂O, 2% BSA, and 0.1% glucose] to a concentration of ~100 CFU in 60 μL. Serum samples from individual mice were pooled, heat-inactivated (56 °C, 30 min), and diluted 10-fold serially in PBS. The assay condition was consisted of 60 μL of bacillus cell suspension, 20 μL of heat-inactivated serum, and 20 μL of human complement (Sigma, diluted 1:4 in PBS). The mixture was incubated at 37 °C for 1 h without shaking. Samples from each incubation mixture (50 μL) were plated on LB agar plates, and the plates were incubated for 8 h at 37 °C. The number of colonies formed was determined. As controls, bacteria were incubated with human complement alone or with PBS alone before being plated onto the LB agar plates. The percent of killed bacterial cells was calculated by comparing with the number of colonies formed when the bacteria were incubated with complement alone.

**In vivo LeTx challenge study**

To preliminarily evaluate the extent to which nasal immunization with the mixture of rPA63, LF, and PGA–BSA conjugate admixed with poly(I:C) can protect the mice against anthrax LeTx challenge, an *in vivo* LeTx challenge study was completed. Two weeks after the third immunization, mice were injected via the tail vein with the mixture of rPA83 (90 μg) and LF (45 μg) (i.e. 7.5 × LD₅₀) (Aulinger et al., 1997). As a control, mice were injected with complement alone.
et al., 2005). Mice were monitored several times daily for 2 weeks. As controls, unimmunized mice and mice nasally immunized with rPA63 alone admixed with poly(I:C) or with LF alone admixed with poly(I:C) were also challenged similarly. At the conclusion of the 2-week observational period, all surviving mice were euthanized and bled by cardiac puncture to collect serum samples. The anti-PA83 IgG titer, the anti-LF IgG titer, and the lethal TNA in the serum samples were determined.

Statistics
Statistical analyses were completed using ANOVA followed by the Fischer’s protected least significant difference procedure. A P-value of \( \leq 0.05 \) (two tail) was considered to be statistically significant.

Results

The optimal nasal dose of poly(I:C) was determined to be 10 \( \mu \text{g} \) in mice

To identify the optimal dose of nasal poly(I:C), mice were nasally dosed with the rPA83 admixed with various amounts of poly(I:C). As shown in Fig. 1a, mice nasally dosed with the rPA83 admixed with 0.1 or 1.0 \( \mu \text{g} \) of poly(I:C) failed to induce adequate anti-PA83 IgG Abs. However, mice nasally dosed with the rPA83 admixed with 10 \( \mu \text{g} \) of poly(I:C) induced a significantly higher level of anti-PA IgG Abs, when compared with untreated mice. Further increasing the dose of the poly(I:C) from 10 to 20 or 40 \( \mu \text{g} \) did not lead to any further increase in the resultant anti-PA83 IgG level (Fig. 1a). Therefore, all following experiments were completed using 10 \( \mu \text{g} \) of poly(I:C) per mouse. Data in Fig. 1b showed that the nasal poly(I:C) did not induce any detectable level of anti-dsRNA Abs in mouse serum samples.

Nasal immunization of mice with the LF induced functional anti-LF Abs

To evaluate the feasibility of inducing anti-LF Abs by nasal LF, mice were nasally dosed with various amounts of LF adjuvanted with poly(I:C). As shown in Fig. 2a, nasal LF induced anti-LF IgG in the serum samples. Increasing the dose of the LF tended to increase the resultant anti-LF IgG levels (LF dose vs. anti-LF IgG titer, \( R^2 = 0.89 \)). Moreover, data in Fig. 2b showed that the anti-LF Abs have anthrax lethal TNA.

Nasal immunization with the mixture of rPA83, LF, and a \( \gamma \text{DPGA} \) conjugate induced anti-PA, anti-LF, and anti-\( \gamma \text{DPGA} \) Ab responses

Shown in Table 1 are the anti-PA83, anti-LF, and anti-\( \gamma \text{DPGA} \) Ab titers in the serum samples when the mice were nasally immunized with the physical mixture of PA83, LF, and the \( \gamma \text{DPGA} \) conjugate using the poly(I:C) or the CT as an adjuvant. Apparently, nasal immunization with the mixture of those three antigens induced anti-PA, anti-LF,
and anti-\(\gamma\)DPGA Abs, and the Ab responses were biased toward IgG1 (Table 1). The anti-LF IgG titer was relatively weaker, probably due to the low dose of the LF administered. In addition, the splenocytes isolated from the immunized mice also proliferated significantly after in vitro restimulation with rPA83 or LF (Fig. 3a and b), although proliferation was not detectable when restimulated with the \(\gamma\)DPGA.

Finally, theAbs (both anti-PA and anti-LF) induced were able to neutralize anthrax lethal toxin, and thus, protect mouse macrophages (J774A.1) against a lethal toxin challenge in vitro (Fig. 3c). Similarly, the anti-\(\gamma\)DPGA Abs induced were also able to inhibit the growth of the \(\gamma\)DPGA–producing freshly germinated vegetative \(B.\ licheniformis\) bacterial cells by complement-mediated bacteriolysis (Fig. 3d).

Nasal immunization with the mixture of rPA63, LF, and the \(\gamma\)DPGA conjugate induced Ab responses against all three antigens

In this experiment, the feasibility of using the PA63, instead of the PA83, in the antigen mixture was evaluated. Moreover, to enhance the anti-LF Abs induced, the dose of the LF in the antigen mixture was increased from 5 to 20 \(\mu\)g mouse\(^{-1}\). Data in Fig. 4a showed that nasal immunization of mice with the mixture of rPA63, LF, and \(\gamma\)DPGA–BSA induced anti-PA, anti-LF, and anti-PGA Abs. The specific Abs induced were comparable to that in mice s.c. injected with the antigen mixture adjuvanted with Alum (Fig. 4a). In addition, the levels of the serum anti-PA83 Abs induced by the nasal rPA83 and nasal rPA63 were comparable (\(P = 0.07\)) (Fig. 4a).

The Abs induced by this triantigen nasal vaccine candidate were able to protect all mice (\(n = 4\)) against a lethal dose (7.5 \(\times \) LD\(_{50}\)) of anthrax LeTx challenge (Table 2). Nasal rPA63 alone adjuvanted with poly(I:C) or LF alone adjuvanted with poly(I:C) both provided protection too (Table 2). However, one mouse in each of those two groups died 5 days after the challenge, and another mouse in each group showed signs of LeTx intoxication, although they slowly recovered by the end of the study. Also, one mouse that was immunized with the LF alone adjuvanted with poly(I:C) failed to regain consciousness after the injection of the LeTx. As expected, all unimmunized mice (\(n = 5\)) died within 36 h after the LeTx challenge.

Finally, the levels of anti-PA83 and anti-LF Abs in the postchallenge serum samples from mice nasally immunized

<table>
<thead>
<tr>
<th>Vaccine formulations</th>
<th>Anti-PA IgG ((\text{mean} \pm \text{SD}))</th>
<th>Anti-LF IgG ((\text{mean} \pm \text{SD}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(rPA83+LF+PGA)/pI:C, i.n.</td>
<td>17.3 ± 1.2 (\times) 1.2</td>
<td>16.7 ± 1.5 (\times) 1.5</td>
</tr>
<tr>
<td>(rPA83+LF+PGA)/CT, i.n.</td>
<td>17.5 ± 1.8 (\times) 1.3</td>
<td>17.7 ± 1.3 (\times) 1.3</td>
</tr>
<tr>
<td>Anti-PA IgA</td>
<td>9.4 ± 0.8 (\times) 3.0</td>
<td>8.6 ± 0.8 (\times) 3.0</td>
</tr>
<tr>
<td>Anti-PA IgM</td>
<td>4.6 ± 3.0 (\times) 4.1</td>
<td>7.7 ± 4.1 (\times) 4.1</td>
</tr>
<tr>
<td>Anti-LF IgG</td>
<td>10.2 ± 0.6 (\times) 3.8</td>
<td>15.2 ± 3.8 (\times) 3.8</td>
</tr>
<tr>
<td>Anti-LF IgG1</td>
<td>10.4 ± 1.1 (\times) 3.7</td>
<td>14.3 ± 4.0 (\times) 3.7</td>
</tr>
<tr>
<td>Anti-LF IgG2a</td>
<td>4.6 ± 2.8 (\times) 2.8</td>
<td>11.4 ± 0.5 (\times) 2.8</td>
</tr>
<tr>
<td>IgG1/IgG2a</td>
<td>56</td>
<td>5</td>
</tr>
<tr>
<td>Anti-PGA IgG</td>
<td>13.4 ± 1.1 (\times) 0.6</td>
<td>12.4 ± 1.2 (\times) 0.6</td>
</tr>
<tr>
<td>Anti-PGA IgG1</td>
<td>13.8 ± 1.6 (\times) 0.9</td>
<td>12.4 ± 1.6 (\times) 0.9</td>
</tr>
<tr>
<td>Anti-PGA IgG2a</td>
<td>9.9 ± 1.9 (\times) 1.8</td>
<td>8.6 ± 1.8 (\times) 1.8</td>
</tr>
<tr>
<td>IgG1/IgG2a</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Anti-PGA IgA</td>
<td>7.2 ± 2.2 (\times) 2.1</td>
<td>4.8 ± 2.1 (\times) 2.1</td>
</tr>
<tr>
<td>Anti-PGA IgM</td>
<td>11.1 ± 2.5 (\times) 2.1</td>
<td>8.2 ± 2.1 (\times) 2.1</td>
</tr>
</tbody>
</table>

Titers are in anti-log2 \((\text{mean} \pm \text{SD}, n = 5)\)
with the triantigen vaccine candidate (PA63, LF, and the γDPGA conjugate) were comparable to that in mice nasally immunized with the rPA63 alone or with the LF alone, respectively (data not shown). However, when the serum samples from the mice that survived the challenge were pooled to test their ability to neutralize anthrax lethal toxin using the in vitro macrophage protection assay, the serum samples from mice nasally immunized with the triantigen vaccine candidate increased the NC50 (neutralizing concentration) of the anthrax LeTx by 1.7-fold when compared with that from mice immunized with the rPA63 alone as the antigen \((P = 0.02)\), and by nine-fold when compared with that from mice immunized with the LF alone as the antigen \((P = 0.001)\) (Fig. 4b).

Discussions

The present study has three principal findings. (1) The optimal dose of poly(I:C) as a nasal vaccine adjuvant to be \(10 \mu g \text{mouse}^{-1}\) was identified, and it was shown that the poly(I:C) did not induce any detectable anti-dsRNA Abs. (2) It was shown that nasal immunization of mice with LF induced functional anti-LF Abs. (3) It was demonstrated that a prototypic triantigen vaccine candidate consisted of rPA63, LF, and a γDPGA conjugate induced Abs against all three antigens and protected the mice against a lethal dose of anthrax LeTx challenge.

Data from several studies have shown that anti-PA Abs alone did not provide the optimal protection against inhalation anthrax (Little & Knudson, 1986; Turnbull et al., 1986; Ivins et al., 1998; Brossier et al., 2002). It was thus thought that an efficacious anthrax vaccine should be able to rapidly induce immune responses against both virulence factors, the capsule, by which vegetative bacillus cells are encapsulated, and the anthrax toxins (Wang & Roehrl, 2005). The AVA and the new generation anthrax vaccines currently under development are primarily based on the anthrax PA protein alone. However, the level of anthrax toxin neutralizing Abs required to confer immunity to \(B.\ anthracis\) is expected to be high and may be difficult to induce and maintain if a large number of spores are inhaled (Dixon et al., 1999; Inglesby et al., 2002). Thus, the addition of anti-γDPGA Ab-mediated opsonophagocytic killing of vegetative bacillus cells is expected to prevent or inhibit bacterial replication and augment the protection mediated by anthrax toxin neutralizing Abs. This is expected to be particularly true in the case of a postexposure combination therapy of anthrax with a vaccine and an antibiotic. In addition, the PA serves

Fig. 3. Nasal immunization of mice with the mixture of rPA83, LF, and γDPGA–BSA adjuvanted with poly(I:C) induced anti-PA, anti-LF, and anti-γDPGA immune responses. (a) Splenocyte proliferation after in vitro restimulation with rPA. The proliferation indices of the two treated groups were significantly different from each other \((P = 0.003)\) and greater than that of the untreated mice \((P = 0.001)\). (b) Splenocyte proliferation after in vitro restimulation with LF. The proliferation indices of the two treated groups were significantly different from each other \((P = 0.001)\) and greater than that of the untreated mice \((P = 0.001)\). (c) The Abs (anti-PA and anti-LF) protected macrophage cells against anthrax lethal toxin in vitro. (d) The anti-γDPGA Abs had complement-mediated bacteriolytic activity against \(Bacillus licheniformis\). The activity of the untreated control was set to zero. All data reported are mean ± SD \((n = 3)\).
as a carrier to facilitate the entry of LF and EF into the host cell cytosol (Bradley et al., 2001). Thus, a vaccine that can induce both anti-PA and anti-LF Abs is expected to provide a better protection than vaccines consisting of PA alone, and there were previous data that were supportive of this (Ivins & Welkos, 1988).

There have been many attempts to design an anthrax vaccine to target both the anthrax toxins and the vegetative bacilli. For example, it was reported that s.c. injection of rPA83 conjugated with a PGA peptide or protein induced both anti-PA and anti-PGA immune responses (Schneerson et al., 2003; Wang et al., 2004). Similarly, Wimer-Mackin et al. (2006) reported that nasal immunization of rabbits with a rPA83 protein–PGA peptide conjugate generated both anti-PA and anti-PGA Abs (Wimer-Mackin et al., 2006). However, no efforts were directed toward evaluating the extent to which the induction of anti-PGA Abs would further improve the resultant protective activity against anthrax spore challenges. Likewise, there have been several recent studies where both PA and LF were dosed together as antigens in an anthrax vaccine (Ivins & Welkos, 1988; Price et al., 2001; Galloway et al., 2004; Hermanson et al., 2004), and a possible synergistic effect was suggested for the resultant LeTx neutralization activity. For example, it was reported that when guinea pigs were vaccinated with PA in combination with LF, 81% of them survived an anthrax spore challenge, whereas only 69% of guinea pigs vaccinated with PA alone as the antigen survived, indicating that vaccination with PA and LF together afforded a greater protection than with the PA alone (Ivins & Welkos, 1988). The in vitro anthrax lethal toxin neutralization data shown in Fig. 4b supported this. Therefore, it is expected that a triantigen anthrax vaccine, such as the one proposed, will provide a more robust protection against an anthrax infection, particularly when used for postexposure prophylaxis. More experiments have to be completed in future studies to confirm this.

In the present study, the anti-PA and anti-LF Abs induced were able to protect macrophages in an in vitro neutralization assay (Fig. 2c). Additionally, the anti-γDPGA Abs induced were able to inhibit the growth of freshly germinated B. licheniformis bacterial cells (Fig. 2d). It is likely that the complement-mediated bacteriolysis assay used in the present study more accurately reflected that of a natural infection than a similar assay previously used by the authors (Sloat & Cui, 2006a), in which the bacillus cells in their exponential proliferative growth stage were used. In the exponential proliferative stage, the bacteria also shed their PGA. It is worthy to point out that the anti-γDPGA Abs induced in this study may or may not be protective against B. anthracis (Chabot et al., 2004; Kozel et al., 2004; Joyce et al., 2006), and future experiments using B. anthracis strain need to be completed to confirm this.

In the present study, the poly(I:C), a synthetic dsRNA, was used as the nasal mucosal vaccine adjuvant. Poly(I:C) was known to have adjuvant activity (Park & Baron, 1968;
Giantonio et al. (PA83 or PA63), LF, and a prototypic anthrax vaccine candidate that contained PA preclinical and clinical trials. Evaluate the safety of nasal poly(I:C) as an adjuvant in future studies, the present vaccine candidate is expected to represent one of the three antigens after only two or three intranasal doses. When repeatedly dosed nasally to mice at 10 μg daily for 9 consecutive days (Ichinohe et al., 2005). Nasal poly(I:C) was shown to upregulate the expression of the TLR3 and alpha/beta interferons, as well as T helper type I (Th1)- and Th2-related cytokines (Ichinohe et al., 2005). In a preliminary preclinical safety study, poly(I:C) did not generate any detectable side-effects when repeatedly dosed nasally to mice at 10 μg daily for 9 consecutive days (Ichinohe et al., 2005). Additionally, intracerebral injection of poly(I:C) at a dose as high as 25 μg/mouse did not lead to any detectable toxicity in mouse brain tissues (Ichinohe et al., 2005). Finally, this study also demonstrated that poly(I:C) did not induce any detectable levels of anti-dsRNA Abs in mouse serum samples (Fig. 1b). All these preclinical data suggested that the poly(I:C) is safe when used as a nasal vaccine adjuvant. In fact, the safety profile of poly(I:C) dosed by other routes had been previously investigated in several clinical trials (Cornell et al., 1976; Guggenheim & Baron, 1977; Levine et al., 1979; Bever et al., 1986; Salazar et al., 1996; Giontonio et al., 2001). It was shown to be generally safe, although repeated high doses (e.g. i.p. injection) tended to induce some side effects, including fever and abnormal liver function (Cornell et al., 1976; Robinson et al., 1976; Guggenheim & Baron, 1977; Levine et al., 1979; Bever et al., 1986; Salazar et al., 1996; Giontonio et al., 2001). However, it can be speculated that when used as a vaccine adjuvant and at a relatively low dose for 2 or 3 times only, nasal poly(I:C) is unlikely to generate severe side effects. More experiments will be carried out to evaluate the safety of nasal poly(I:C) as an adjuvant in future preclinical and clinical trials.

In conclusion, the data in this study showed that a prototypic anthrax vaccine candidate that contained PA (PA83 or PA63), LF, and a γDPGA–carrier protein conjugate was able to induce strong Ab immune responses against all three antigens after only two or three intranasal doses. When fully optimized, a triantigen nasal vaccine similar to the present vaccine candidate is expected to represent one of the newer generation anthrax vaccines. In future studies, the γDPGA will be directly conjugated to PA and a detoxified mutant LF will be used to validate the efficacy of the resultant immune responses in protecting mice against an inhalational anthrax spore challenge.

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