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Research Paper

Nasal Immunization with Anthrax Protective Antigen Protein Adjuvanted with Polyriboinosinic–Polyribocytidylic Acid Induced Strong Mucosal and Systemic Immunities

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Purpose. The current anthrax vaccine adsorbed (AVA) was originally licensed for the prevention of cutaneous anthrax infection. It has many drawbacks, including the requirement for multiple injections and subsequent annual boosters. Thus, an easily administrable and efficacious anthrax vaccine is needed to prevent the most lethal form of anthrax infection, inhalation anthrax. We propose to develop a nasal anthrax vaccine using anthrax protective antigen (PA) protein as the antigen and synthetic double-stranded RNA in the form of polyriboinosinic–polyribocytidylic acid (pI:C) as an adjuvant.
Methods. Mice were nasally immunized with recombinant PA admixed with pI:C. The resulting PA-specific antibody responses and the lethal toxin neutralization activity were measured. Moreover, the effect of pI:C on dendritic cells (DCs) was evaluated both *in vivo* and *in vitro*.
Results. Mice nasally immunized with rPA adjuvanted with pI:C developed strong systemic and mucosal anti-PA responses with lethal toxin neutralization activity. These immune responses compared favorably to that induced by nasal immunization with rPA adjuvanted with cholera toxin. Poly(I:C) enhanced the proportion of DCs in local draining lymph nodes and stimulated DC maturation.
Conclusions. This pI:C-adjuvanted rPA vaccine has the potential to be developed into an efficacious nasal anthrax vaccine.

KEY WORDS: adjuvant; dendritic cells; double-stranded RNA; toxin neutralization.

INTRODUCTION

Anthrax is an often fatal bacterial infection caused by *Bacillus anthracis* (1). Although cutaneous and gastrointestinal anthrax infections have produced documented fatalities, inhalation anthrax has a fatality rate close to 100%, with death occurring shortly after the onset of symptoms (2). Under adverse conditions, anthrax bacteria form endospores, which are sufficiently small enough for inhalation and deposition in the alveolar spaces of the host respiratory tract (3). Anthrax is a toxin-mediated disease. Anthrax toxin contains three components, the protective antigen (PA), lethal factor (LF), and edema factor (EF). Although none of these components alone is toxic, they can combine to form two binary toxins, the lethal toxin (LeTx, PA + LF) and the edema toxin (PA + EF). PA binds to the anthrax toxin receptor on cell surface and mediates the entry of LF and EF into host cell cytosol (4), in where they are toxic (1). Thus, anti-PA antibodies (Abs) that block the transport of LF and EF into cell cytosol have been shown to prevent the course of infection (5).

Anthrax vaccine adsorbed (AVA), an aluminum hydroxide (Alum)-adsorbed, formalin-treated culture supernatant of a toxigenic *B. anthracis* strain, is the only licensed anthrax vaccine currently available in the United States (6). Previous studies demonstrated that the primary antigen component in the AVA was the PA protein. AVA was originally licensed only for the prevention of cutaneous anthrax. Although it was shown to be effective against inhalational anthrax infection in many animal models, including nonhuman primates (7), its efficacy against an inhalational anthrax infection in humans has yet to be confirmed. Moreover, AVA suffers from a lengthy dosing schedule with six initial injections in the first year, followed by annual boosters (8,9). Thus, there continues to be a need for developing alternative and improved anthrax vaccines to effectively prevent against anthrax infection.

A nasal anthrax vaccine would be advantageous not only because it will be easily administrable, but also because it is expected to induce systemic immune responses, as well as PA-specific mucosal immune responses in the respiratory tract, through which inhaled anthrax spores enter hosts (2,3). Therefore, it is conceivable that the immune response induced by a nasal PA-based anthrax vaccine will be more effective in preventing an inhalational anthrax infection than a systemically injected rPA-based vaccine. Anti-PA Abs have been shown to recognize spore-associated proteins, to stimulate spore uptake by macrophages, and to interfere

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with the germination of spores *in vitro* (10–12), suggesting that anti-PA Abs in the respiratory mucosa will exert a certain extent of antispore activity. In support of this concept, it was recently reported that in rabbits nasally immunized with PA protein adjuvanted with CpG oligos, serum LeTx neutralization Ab titers alone were not predictive of the survival of rabbits after a pulmonary anthrax spore challenge (13), which is in sharp contrast to the previous belief that serum LeTx neutralization Ab titers alone were predictive of the efficacy of an anthrax vaccine (5). Similarly, using rPA-loaded polylactic-*co*-glycolic acid (PLGA) microspheres, it was reported that mice that were immunized by a combination of intramuscular (i.m.) and nasal priming and boosting schedules were always fully protected against aerosol anthrax spore challenges, whereas i.m. injection of the PA-loaded microspheres alone was not as effective (14). In fact, there had been a few more reports on the evaluation of immune responses induced by nasally administered PA protein. Although nasal PA protein alone failed to induce any anti-PA immune responses, nasal PA was immunogenic when it was incorporated into liposomes (15) or a liposome-based system (16), or when cholera toxin (CT) was used as a mucosal adjuvant (17).

Double-stranded RNA is produced by many viruses during their replicative cycle (18,19). It was known in the 1960s–1970s that poly(I:C), a synthetic dsRNA, can boost immune responses (20–24). However, it was only recently found that dsRNA functioned through TLR3 (25), which stimulated more interest in the study of the adjuvanticity of dsRNA. Poly(I:C) was recently shown to induce stable maturation of DCs (26), to activated natural killer (NK) cells (27,28), to promote the survival of activated $CD4^+$ T cells *in vitro* (29), and to boost the number of antigen-specific $CD8^+$ T cells and their survival *in vivo* (30). More recently, pI:C was demonstrated to enhance the immune responses against nasally dosed vaccines too. For example, Ichinohe *et al.* (31) reported that nasal immunization of mice with an inactivated hemaglutinin (HA) admixed with pI:C protected the mice against an influenza viral infection (31). Similarly, Partidos *et al.* (32) reported the induction of specific serum and mucosal Abs, as well as cellular responses, to the HIV TAT protein when the molecular complex of TAT and pI:C was nasally administered into mice (32).

In this present study, we evaluated the anti-PA immune responses induced by nasal immunization of mice with rPA admixed with pI:C as an adjuvant, hypothesizing that pI:C is a strong mucosal adjuvant for PA protein. We reported that the nasally dosed rPA adjuvanted with pI:C induced strong mucosal and systemic anti-PA immune responses. Moreover, pI:C enhanced the proportion of DCs in local draining lymph nodes (LNs) and stimulated the maturation of DCs.

MATERIALS AND METHODS

Nasal Immunization

All animal studies were carried out by following NIH guidelines for animal use and care. Poly(I:C) (10, 20, or 40 μg/mouse; GE Amersham Biosciences, Piscataway, NJ, USA) and rPA (5 μg/mouse; List Biological Laboratories, Campbell, CA, USA) were dissolved into phosphate-buffered saline (PBS, 10 mM, pH 7.4; Sigma-Aldrich, St. Louis, MO, USA). Mice (n = 5) were lightly anesthetized (i.p., pentobarbital, 6 mg/100 g; Abbott Laboratories, North Chicago, IL, USA) and given a total volume of 20 μL of rPA/pI:C solution in two 10-μL doses, with 10–15 min between each dose, half in each nare. As controls, mice (n = 5) were subcutaneously (s.c.) injected with rPA (5 μg/mouse) admixed with aluminum hydroxide gel (Alum, 15 μg/mouse, USP grade; Spectrum Chemical and Laboratory Products, New Brunswick, NJ, USA), nasally dosed with rPA admixed with cholera toxin (CT, 1 μg/mouse; List Biological Laboratories) as a mucosal adjuvant, or left untreated. Mice were dosed on days 0, 7, and 14, bled via the tail vein on days 21 and 30, and euthanized on day 30 by using CO_2. This experiment was repeated independently twice.

The pI:C was a duplex RNA polymer composed of a poly(I) strand and a poly(C) strand. According to GE Amersham product information, the two strands were synthesized independently and then annealed together, which resulted in extensive heterogeneity in duplex lengths. The length of poly(I) and poly(C) was 152–539 and 319–1305 bases, respectively. pI:C was believed to be primarily double-stranded in nature, although some single-stranded regions may exist. The endotoxin level in our pI:C solution was determined to be 2.4 ± 0.3 EU/mL by using a Limulus Lysate

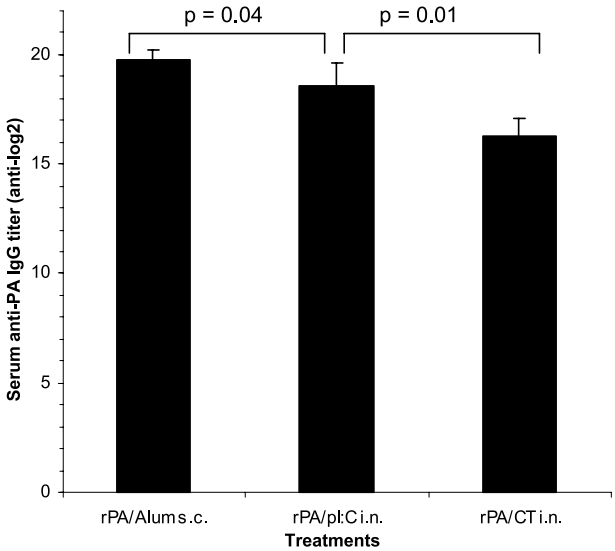


Fig. 1. Serum anti-PA Ab responses. Balb/C mice (n = 5) were nasally dosed with rPA alone, rPA adjuvanted with pI:C (rPA/pI:C), or left untreated on days 0, 7, and 14. As controls, other two groups of mice (n = 5) were nasally dosed with rPA adjuvanted with CT (rPA/CT i.n., CT = 1 μg/mouse) or s.c. injected with rPA adjuvanted with Alum (rPA/Alum s.c., Alum = 15 μg/mouse). The dose of rPA and pI:C was 5 and 10 μg per mouse, respectively. On day 30, mice were bled, and total anti-PA IgG titer in their serum was determined using ELISA. The sera were diluted 2-fold serially. Nasal rPA alone failed to induce any detectable level of anti-PA IgG. ANOVA analysis showed that there were significant differences among those three treatments ($p \ll 0.05$). This experiment was repeated twice, and similar results were obtained. Data from one representative were shown (mean ± SD, n = 5).

Assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA).

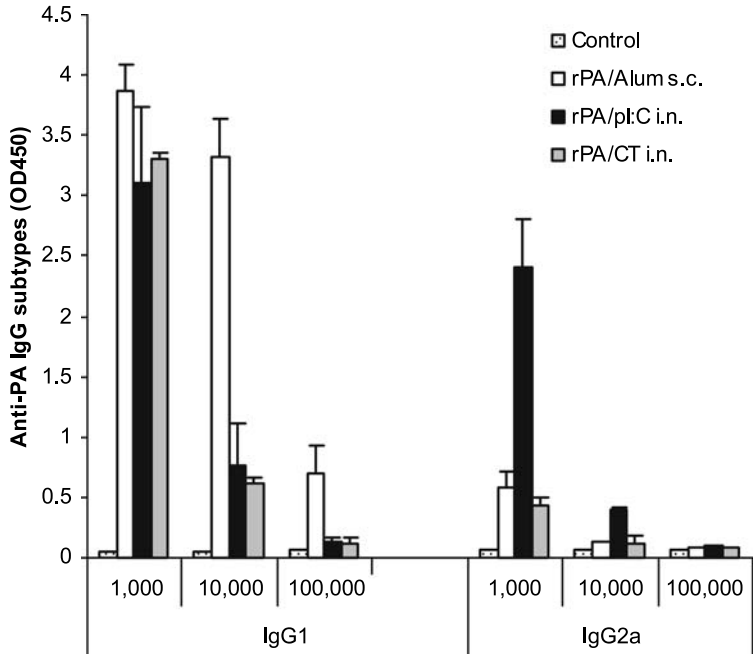
Bronchoalveolar Lavage Collection

Mice were euthanized 30 days after the first immunization. To collect the bronchoalveolar lavages (BALs), an incision was made in the trachea; care was taken to avoid cutting the blood vessels close to the trachea. A 0.4-mL sterile PBS was pipetted through the trachea toward the lungs, aspirated back into the pipette tip, and reinjected once before the final withdrawal of the PBS solution. Lavage samples collected from each mouse were stored at −80°C prior to further use.

ELISA for Anti-PA Ab Measurement

Enzyme-linked immunosorbent assay (ELISA) was used to determine the anti-PA Ab levels. Briefly, EIA/RIA flat-bottom, medium-binding, polystyrene 96-well plates (Corn-

A



B Ratio of IgG1 over IgG2a ($OD450_{IgG1}/OD450_{IgG2a}$)

	1,000	10,000	100,000
Untreated	0.8	0.8	0.8
rPA/Alum s.c.	6.6	25.6	8.7
rPA/pI:C i.n.	1.3	1.9	n/a
rPA/CT i.n.	7.6	5.4	n/a

Fig. 2. Serum anti-PA IgG subtypes. Balb/C mice (n = 5) were dosed with rPA alone, rPA/pI:C, or left untreated on days 0, 7, and 14. As controls, other groups of mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. The IgG1 and IgG2a levels in the sera were determined on day 30 using ELISA after the serum samples were diluted 1,000-, 10,000-, and 100,000-fold. (A). Data shown were mean ± SD (n = 5). (B) The ratio of the OD450 for IgG1 and IgG2a ($OD450_{IgG1}/OD450_{IgG2a}$). N/A, for rPA/pI:C (i.n.) and rPA/CT (i.n.), values in the 100,000-fold dilution were not reported because their IgG1 values were not significantly different from that of the untreated mice. Nasal rPA alone failed to induce any detectable level of anti-PA IgG subtypes.

ing Costar, Corning, NY, USA) were coated with 100 ng rPA dissolved in 100 μL carbonate buffer (0.1 M, pH = 9.6) overnight at 4°C. Plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20, Sigma) and blocked with 4% (w/v) bovine serum albumin (BSA, Sigma) in PBS/Tween 20 for 1 h at 37°C. Serum and BALs samples were diluted (2-fold serial except where mentioned) in 4% BSA/PBS/Tween 20, added to the plates following the removal of the blocking solution, and incubated for an additional 2.5 h at 37°C. The samples were removed, and the plates were washed five times with PBS/Tween 20. Horseradish peroxidase (HRP)-labeled goat-antimouse immunoglobulin (IgG, IgG1, IgG2a, IgM, or IgA, 5000-fold dilution; Southern Biotechnology Associates, Inc., Birmingham, AL, USA) was added as the secondary Ab into the plates, followed by a 1-h 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), followed by the addition of sulfuric acid (0.2 M; Sigma) as the stop solution. The absorbance was read at 450 nm by using a SpectraMax Plate reader (Molecular Devices Inc., Sunnyvale, CA).

Lethal Toxin Neutralization Activity (TNA) Assay

Toxin neutralization activity (TNA) was determined as described elsewhere with slight modifications (16,17). Briefly, confluent J774A.1 cells were plated (5.0×10^4 cells/well) in sterile, 96-well clean-bottom plates (Corning Costar) and incubated at 37°C, 5% CO_2 for 16 h. A fresh solution (50 μL) containing rPA (400 ng/mL) and LF (40 ng/mL) was mixed with 50 μL diluted serum or BAL samples in triplicate and incubated for 1 h at 37°C. The cell culture medium was removed, and 100 μL serum/LeTx mixture was added to each well and incubated at 37°C, 5% CO_2 for 3 h. Cell viability was determined by using an MTT assay kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; Sigma-Aldrich) with untreated or LeTx (alone)-treated cells as controls.

Splenocyte Proliferation Assay

Single cell suspensions from individual spleens were prepared as previously described (33). Splenocytes were cultured at a density of 4×10^6 cells/mL with rPA (0 or 12.5 μg/mL) in complete RPMI medium (Invitrogen, Carlsbad, CA, USA) for 5 days at 37°C, 5% CO_2 (17). Cell proliferation was determined by using an MTT kit. Proliferation index was reported as the ratio of the final number of cells after stimulation with rPA (12.5 μg/mL) over that without stimulation (0 μg/mL).

Effect of Nasal pI:C on DCs in Local Draining Lymph Nodes

Balb/C mice ($n = 3$) were lightly anesthetized, and then nasally dosed with 20 μL sterile PBS, pI:C (20 μg, 1 mg/mL), or CT (1 μg) as described above. Twenty hours later, mice were euthanized, and their superficial and deep cervical LNs were removed. As controls, mice ($n = 3$) were subcutaneously injected in their hind leg footpads with PBS alone or lipopolysaccharide in PBS (LPS, 100 ng; List Biological Laboratories), and their draining popliteal LNs were removed 20 h later. The LNs from mice with the same treatment were pooled and incubated with collagenase (1 mg/mL; Sigma) for 20 min at 37°C, followed by a further 10-min incubation at room temperature after the incubation medium was supplemented with EDTA (10 mM; Sigma). Single LN cell suspension was prepared by forcing the LNs through a BD cell strainer (70 μm), stained with FITC-labeled antimouse CD11c Ab (1 μg/2 $\times 10^6$ cells; BD Pharmingen, San Diego, CA) for 20 min at 4°C, washed twice, and analyzed by using a flow cytometer (FC500 Beckman Coulter EPICS V Dual Laser Flow Cytometer; Beckman Coulter, Fullerton, CA).

CD80 and CD86 Expression and TNF-α Secretion by DCs

Bone morrow-derived DCs (BMDCs) were isolated as previously described (34). Briefly, bone marrow cells from the tibia and femur bones of C57BL/6 mice were depleted of lymphocytes (CD4, CD8, and NK cells) and cultured at a density of 5×10^5 cells/mL in 10% FBS-containing RPMI 1640 with GM-CSF and IL-4 (10^3 U/mL each; R&D Systems, Minneapolis, MN, USA). Loosely adherent cells were collected on day 5. Over 90% of these cells were $CD11c^+$, as confirmed by flow cytometry. To determine the expression of CD80 and CD86 on the surface of DCs, BMDCs (2×10^6) were incubated with pI:C (50 μg/mL), LPS (100 ng/mL), or PBS for 16 h. Next, the cells were stained with FITC-labeled antimouse CD80 and PE-labeled antimouse CD86 (Pharmingen) and analyzed by flow cytometry. Moreover, the TNF-α content in the BMDC culture supernatant was measured by using a TNF-α ELISA kit from BD Pharmingen.

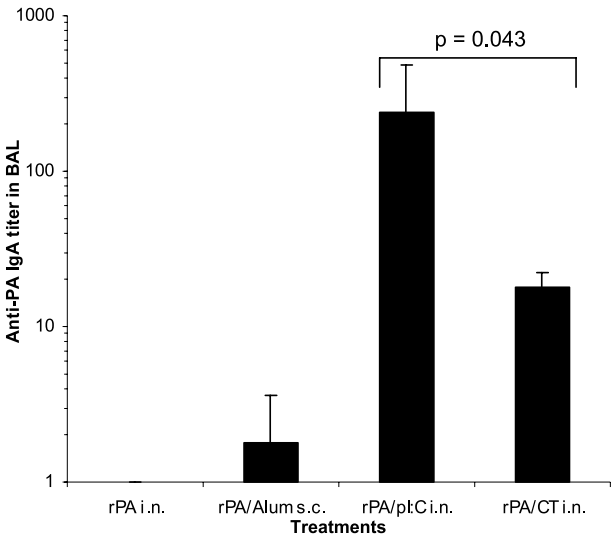


Fig. 3. Nasal immunization with rPA admixed with pI:C induced anti-PA IgA in the BALs of immunized mice. Balb/C mice were dosed (i.n.) with rPA alone, rPA/pI:C, or left untreated on days 0, 7, and 14. As controls, mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. On day 30, mice were euthanized, and their BALs were collected. Anti-PA IgA titer in the BAL samples from individual mouse was determined using ELISA. No anti-PA IgA was detected in the BALs of mice nasally dosed with rPA alone. Statistical analyses showed that the value of rPA/pI:C (i.n.) was significantly different from that of rPA/CT (i.n.) ($p = 0.043$, two-tail t test).

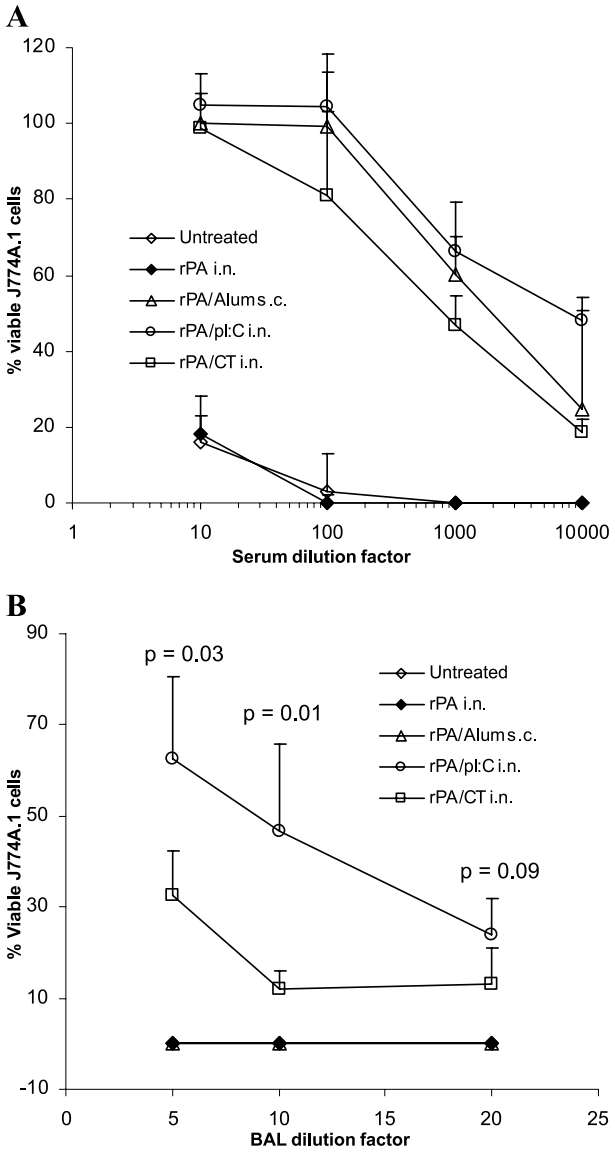


Fig. 4. Nasal immunization with rPA adjuvanted with pI:C induced strong LeTx neutralization activity (TNA) in the serum (A) and BAL (B) of immunized mice. Balb/C mice (n = 5) were dosed (i.n.) with rPA alone, rPA/pI:C, or left untreated on days 0, 7, and 14. As controls, mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. On day 30, serum was collected. The 10-fold serial dilutions of each serum samples were incubated with J774A.1 cells (5×10^5/mL) in the presence of LeTx (400 ng/mL of PA and 40 ng/mL of LF) for 3 h. The number of viable J774A.1 cells was determined using an MTT kit. BAL samples were diluted 5, 10, and 20-fold. (A) Values from rPA/Alum (s.c.), rPA/pI:C (i.n.), and rPA/CT (i.n.) were comparable, although the neutralization activity in the serum of mice nasally immunized with rPA/CT tended to be weaker. (B) TNA was not detectable in the BALs of untreated mice, mice nasally immunized with rPA alone, and mice injected (s.c.) with rPA adjuvanted with Alum. LeTx neutralization activity in the BALs of mice nasally immunized with rPA/pI:C was significantly higher than that in mice immunized with rPA/CT (i.n.) in the 5- and 10-fold dilutions.

Statistics

Statistical analyses were completed by using one-way analysis of variance (ANOVA), followed by the Fischer's protected least significant difference (PLSD) procedure. A p value of ≤ 0.05 (two-tail) was considered to be statistically significant.

RESULTS

Nasal Immunization of Mice with pI:C-Adjuvanted rPA Induced Anti-PA Abs in their Serum

As previously reported, nasal rPA alone (5 μg/mouse) failed to induce any detectable level of anti-PA IgG in mouse serum. However, nasal immunization of mice with rPA admixed with pI:C elicited a significant serum anti-PA IgG response when measured 30 days after the first immunization (Fig. 1). The serum IgG data shown were when the mice were dosed with 10 μg of pI:C. Increasing the dose of pI:C to 20 and 40 μg/mouse did not lead to a significant increase in the serum anti-PA IgG titers induced (data not shown). Although the anti-PA IgG titer induced in mice nasally immunized with the pI:C-adjuvanted rPA was lower than that in mice s.c. injected with rPA adjuvanted with Alum,

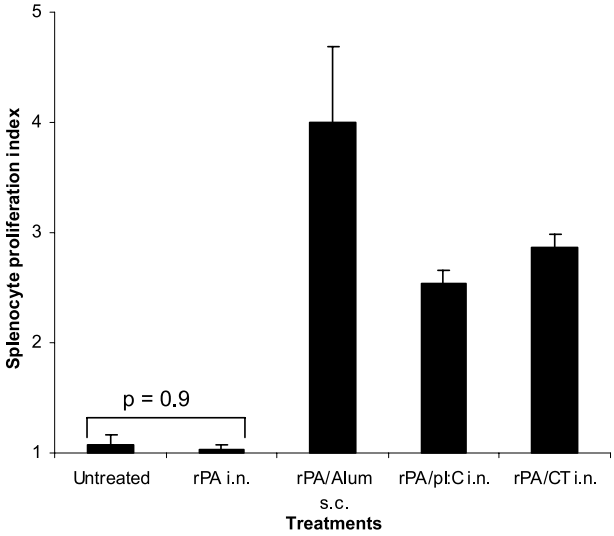


Fig. 5. Splenocytes isolated from mice nasally immunized with rPA adjuvanted with pI:C proliferated after *in vitro* restimulation with rPA. Balb/C mice (n = 5) were dosed (i.n.) with rPA alone, rPA/pI:C, or left untreated on days 0, 7, and 14. As controls, mice were injected (s.c.) with rPA adjuvanted with Alum or dosed (i.n.) with rPA adjuvanted with CT. On day 30, mice were euthanized. Their spleens were harvested, and single splenocyte suspension from individual spleen was prepared. The splenocytes (4×10^6/mL) were incubated with rPA (0 or 12.5 μg/mL) for 5 days. Proliferation (final cell number) was measured using an MTT kit. ANOVA analysis showed that there were significant differences among the values from different treatments ($p \ll 0.05$). Splenocytes isolated from mice nasally immunized with rPA alone did not proliferate (p = 0.9 *vs.* untreated). The values of rPA/Alum (s.c.), rPA/pI:C (i.n.), and rPA/CT (i.n.) were significantly different from each other [rPA/Alum (s.c.) *vs.* rPA/pI:C (i.n.), p = 0.002; rPA/Alum (s.c.) *vs.* rPA/CT (i.n.), p = 0.03; rPA/pI:C (i.n.) *vs.* rPA/CT (i.n.), p = 0.007], and were all higher than that of the untreated mice.

it was higher than that in mice nasally dosed with rPA adjuvanted with CT (Fig. 1). The anti-PA IgG titer induced by the nasal pI:C-adjuvanted rPA was more than 10,000 when measured 21 days after the first immunization. Moreover, although weak, PA-specific IgM was also detected in the serum of mice nasally immunized with rPA adjuvanted with pI:C (data not shown). No significant level of anti-PA IgA was detected in the serum of mice nasally dosed with rPA adjuvanted with pI:C. Based on the levels of anti-PA IgG1 and IgG2a in mouse serum (Fig. 2), the anti-PA immune response induced by the nasal pI:C-adjuvanted rPA was more balanced in contrast to the more IgG1-biased responses induced by s.c. injection of Alum-adjuvanted rPA or nasal immunization with CT-adjuvanted rPA (Fig. 2B). In fact, the anti-PA IgG2a was very weak or not detectable in mice immunized with CT- or Alum-adjuvanted rPA. All these findings demonstrate that nasal rPA adjuvanted with pI:C induced strong anti-PA Abs in mouse serum.

Nasal Immunization of Mice with pI:C-Adjuvanted rPA Induced Anti-PA IgA in Their Lung Mucosal Secretion

Similar to nasal immunization of mice with rPA adjuvanted with CT, nasal rPA adjuvanted with pI:C also induced PA-specific IgA in mouse BALs (Fig. 3). The anti-PA IgA titer in mice nasally immunized with the pI:C-adjuvanted rPA vaccine was, in fact, significantly higher than that induced by the nasal CT-adjuvanted rPA. As expected, s.c. injection of mice with rPA adjuvanted with Alum did not induce any anti-PA IgA in their BALs (Fig. 3). Also, nasal rPA alone failed to induce any anti-PA IgA in mouse BAL samples (Fig. 3).

Anti-PA Antibody Responses Induced by Nasal rPA Adjuvanted with pI:C had Anthrax Lethal Toxin Neutralization Activity

To evaluate the protective activity of the anti-PA Abs induced, the anthrax LeTx neutralization activity in the serum and BAL samples was measured via an *in vitro* macrophage (J774A.1) protection assay. As shown in Fig. 4, LeTx neutralization activity was induced in both the serum and the BALs of mice nasally immunized with rPA adjuvanted with pI:C, but only in the serum (not in the BALs) of mice s.c. injected with rPA admixed with Alum. Although not significant, the LeTx neutralization activity induced by the nasal rPA adjuvanted with pI:C tended to be stronger than that induced by the nasal rPA adjuvanted with CT (Fig. 4A). Moreover, LeTx neutralization activity in the BALs of mice nasally immunized with the pI:C-adjuvanted rPA vaccine was significantly higher than that in mice na-

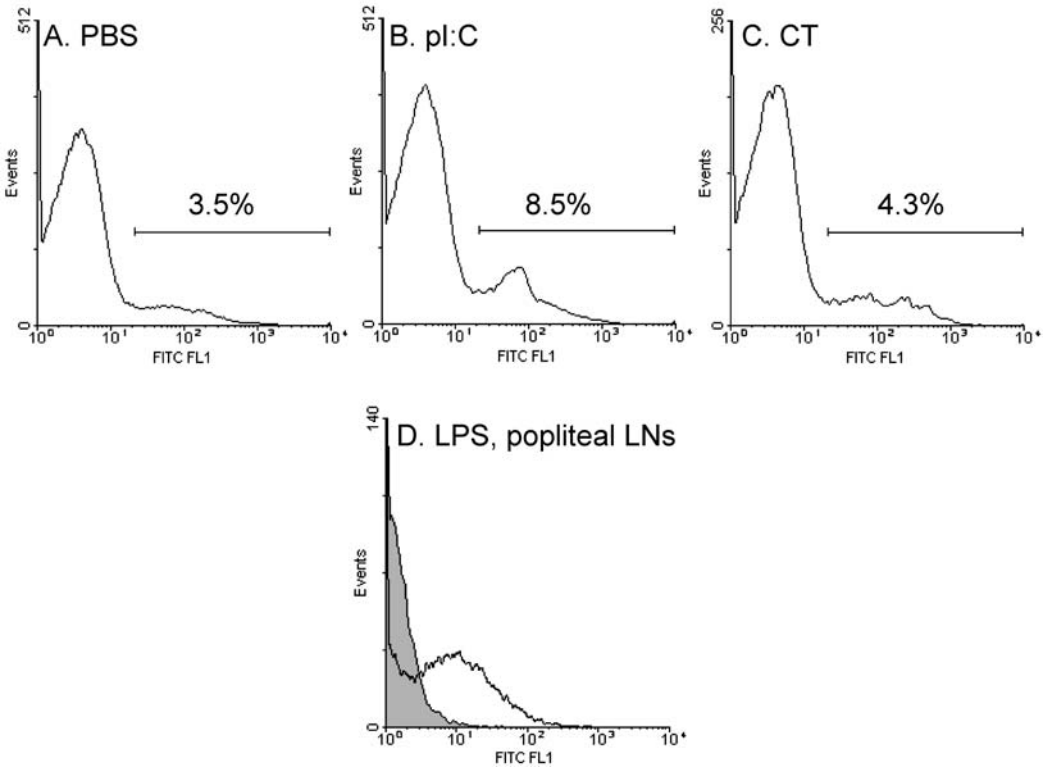


Fig. 6. Nasal pI:C enhanced the proportion of $CD11c^+$ cells in the local draining LNs. Balb/C mice ($n = 3$) were lightly anesthetized, and nasally dosed with 20 μL sterile PBS, pI:C (20 μg), or CT (1 μg). Twenty hours later, mice were euthanized, and their superficial and deep cervical LNs were collected. The LNs from mice with the same treatment were pooled, and single LN cell suspension was prepared. The LN cell samples ($n = 3$) were stained with FITC-labeled antimouse CD11c Ab and analyzed by flow cytometry. Numbers shown above the bars in (A)–(C) represent the mean percent of $CD11c^+$ cells from three independent flow cytometry measurements. (D) As a positive control, mice ($n = 3$) were s.c. injected in their hind leg footpads with LPS (100 ng in PBS) or sterile PBS, and their draining popliteal LNs were removed 20 h later. The graph of the LPS injected mice (dark line) was overlaid on the graph of mice injected with sterile PBS (gray area).

sally immunized with rPA adjuvanted with CT at the 5- and 10-fold BAL dilutions (Fig. 4B). As expected, no LeTx neutralization activity was detected in the BALs of mice s.c. injected with rPA admixed with Alum (Fig. 4B). Finally, LeTx neutralization activity was not detectable in naïve mice and mice nasally dosed with rPA alone.

Splenocytes Isolated from Mice Nasally Immunized with pI:C-Adjuvanted rPA Proliferated After *in Vitro* Restimulation

To evaluate the proliferative immune response induced by nasal immunization of mice with the pI:C-adjuvanted rPA, the splenocytes isolated from the immunized mice were restimulated with rPA, and the final splenocyte number after the restimulation was determined. As shown in Fig. 5, nasal immunization with rPA adjuvanted with pI:C induced a significant splenocyte proliferation. Similarly, s.c. injection of rPA adjuvanted with Alum and nasal dosing of rPA admixed with CT both led to significant splenocyte proliferations (Fig. 5). Again, a proliferative response was not detected in mice nasally dosed with rPA alone without an adjuvant (Fig. 5).

Poly(I:C) Enhanced the Proportion of DCs in Local Draining Lymph Nodes and Stimulated the Maturation of DCs *in Vitro*

To preliminarily elucidate the mechanism responsible for the strong anti-PA immune responses induced by nasal rPA when adjuvanted with pI:C, the effect of pI:C on DCs was evaluated. Nasal pI:C enhanced the proportion of $CD11c^+$ cells in local draining LNs, the superficial and deep cervical

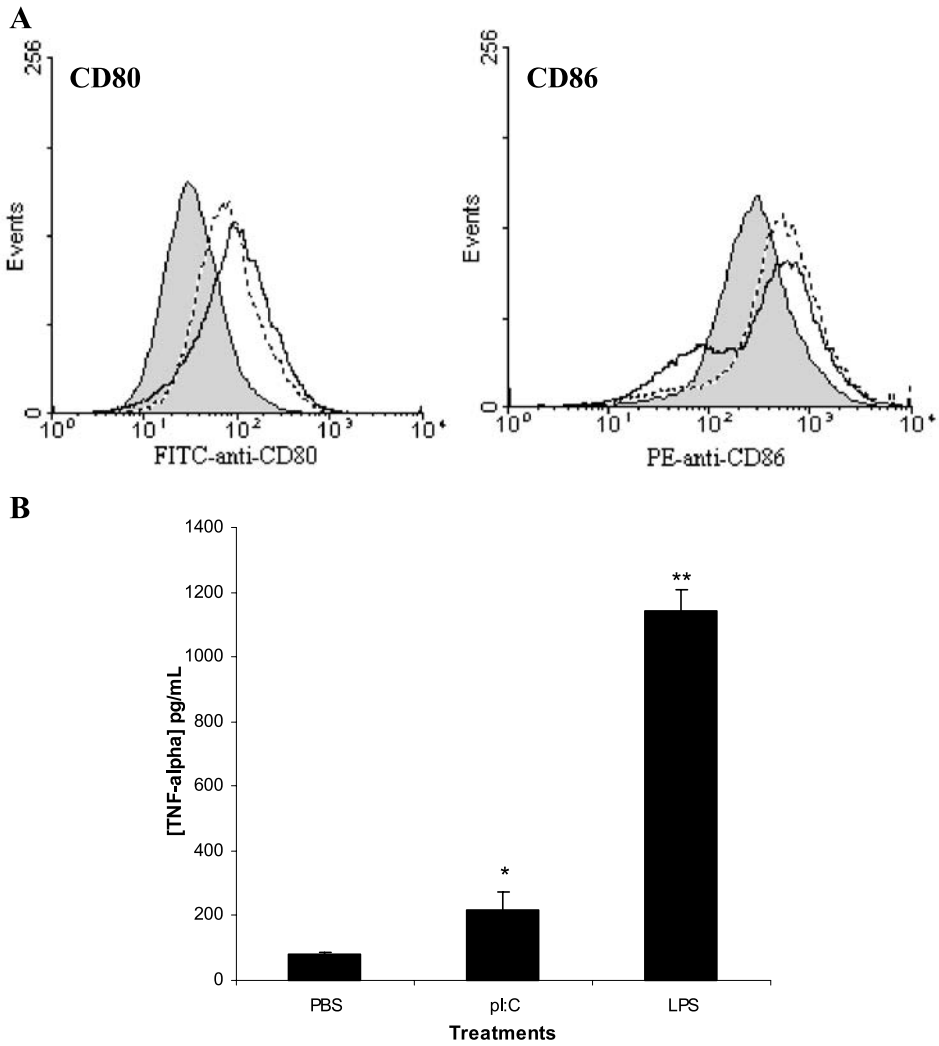


Fig. 7. Poly (I:C) stimulated the expression of CD80 and CD86 on the surface of DCs and the secretion of TNF-α by DCs. (A) BMDCs (2×10^6) isolated from C57BL/6 mice were coincubated with pI:C (50 μg/mL, dash line), LPS (0.1 μg/mL, solid line), or left untreated (gray area) for 16 h at 37°C. Cells were stained with FITC–anti-CD80 and PE–anti-CD86 and analyzed by flow cytometry. Data shown were one representative from three independent studies with similar results. (B) TNF-α content in the cell culture supernatant was measured using ELISA. Data were mean ± SD ($n = 3$). *: Value from pI:C was significantly higher than that from untreated mice ($p = 0.003$, t test); **: Value from LPS was higher than that from the other two treatments ($p \ll 0.05$).

LNs (Fig. 6). *In vitro*, coincubation of BMDCs with pI:C upregulated the expression of DC maturation markers, CD80 and CD86, on the surface of DCs (Fig. 7A) and induced the secretion of TNF-α by the BMDCs into the cell culture supernatant (Fig. 7B).

DISCUSSION

Due to the lengthy dosing schedule of AVA and the uncertainty of its efficacy against an inhalational anthrax infection, we proposed to develop a nasal rPA-based anthrax vaccine. However, to develop such a vaccine, an appropriate mucosal adjuvant is needed because data in published literature had clearly shown that nasal PA alone was unable to induce any detectable PA-specific immune responses (16,17). Using CT and CpG oligos as adjuvants or incorporation of rPA into either liposomes or PLGA microspheres had been previously employed to address this issue (13–17). However, there continues to be a need for alternative nasal mucosal adjuvants. Data shown in this present study indicate that dsRNA, in the form of a synthetic pI:C, is a potential nasal anthrax vaccine adjuvant. When compared to nasal rPA adjuvanted with CT, which is considered by some as a "gold" standard for mucosal adjuvant, nasal rPA adjuvanted with pI:C induced higher anti-PA IgG titers in the serum, higher anti-PA IgA titers in BALs, and more importantly, stronger (or tending to be stronger) anthrax LeTx neutralization activity in the BAL and serum samples (Figs. 1, 3, and 4). All these findings underscored the potential of this pI:C-adjuvanted nasal rPA vaccine. In future studies, we will evaluate the efficacy of the PA-specific immune responses induced by this nasal pI:C-adjuvanted rPA in protecting mice against an inhalational anthrax spore challenge. The fact that the anti-PA IgG titer in the serum of mice nasally immunized with rPA adjuvanted with pI:C was only slightly lower than that in mice s.c. injected with rPA adjuvanted with Alum suggested that nasal rPA adjuvanted with pI:C will be effective in protecting mice against an inhalational anthrax spore challenge (7,13). Moreover, this nasal rPA anthrax vaccine is expected to provide an appropriate model for us to further identify the advantage of the combination of mucosal and systemic anti-PA immune responses over systemic anti-PA immune responses alone in preventing against an inhalational anthrax infection.

As described earlier, dsRNA was established as immunostimulatory back in the 1960–1970s (20,21). But it was only recently that TLR3 was identified as a receptor for dsRNA, which is a pathogen-associated molecular pattern molecule (25). Thus, it is expected that the strong anti-PA immune responses induced by this nasal pI:C-adjuvanted rPA vaccine was related to the dsRNA/TLR3 signaling. We have shown that, when nasally dosed with pI:C, the proportion of $CD11c^+$ cells in the local draining LNs of the dosed mice was significantly increased (Fig. 6). As a positive control, LPS, a ligand for TLR4, when s.c. injected into mouse hind leg footpads, also led to the increase in the proportion of $CD11c^+$ cells in the local draining popliteal LNs (Fig. 6). Although not very specific, CD11c is generally considered to be a DC-restricted marker in mice (35). It is possible that pI:C induced the migration of DCs into local draining LNs. Moreover, as previously reported (25), pI:C induced the maturation of DCs by stimulating the expression of secondary signal molecules, such as CD80 and CD86, on the surface of DCs (Fig. 7A) and the secretion of TNF-α cytokine by DCs (Fig. 7B). These are important because it is known that an antigen presentation without an appropriate secondary signal generally leads to an immune tolerance rather than an immune stimulation (36). Taken together, all these findings suggest that the enhanced specific immune responses against the antigen of interest when the pI:C is used as an adjuvant may be partially attributed to the ability of pI:C to stimulate the migration and maturation of DCs.

Data presented in Fig. 3 indicate that, similar to previous reports (31,32), the Ab response induced by pI:C as an adjuvant was more IgG1/IgG2a-balanced, because both anti-PA IgG1 and IgG2a were induced, in contrast to the more IgG1-biased immune responses induced when Alum or CT were used as adjuvants. For example, the anti-PA IgG1/IgG2a ratio was changed from 0.8 in naïve mice to 6.6–25.6 in mice immunized by s.c injection of rPA admixed with Alum, and to 5.4–7.6 in mice nasally dosed with rPA admixed with CT (Fig. 3B). The more IgG1/IgG2a-balanced anti-PA immune response induced by the nasal rPA adjuvanted with pI:C might hold advantage over an IgG1-biased immune response because it was thought that cellular immune responses, in addition to the PA-specific Ab response, might be beneficial in terms of providing a full protection against anthrax infection in different animal species (9).

Although further experiments have to be completed to precisely define the extent to which the inhalation of the pI:C-adjuvanted PA vaccine into the lungs was responsible for the induction of the anti-PA immune responses, we believe that the involvement of the lungs in the induction of anti-PA immune responses by our nasal pI:C-adjuvanted PA vaccine was limited. It had been shown that the extent to which a nasally dosed vaccine travels into the lungs was largely determined by the volume of the vaccine given to mice (37). It was previously reported that when 50 μL or more of a particle suspension was dosed to anesthetized mice, the majority of it traveled into the lungs. However, when the dose was decreased to 10 μL, they mainly remained in the nare passages (38). Our previous study also showed that when an FITC-labeled liposome suspension was nasally administered to mice by using the same procedure used in this study (i.e., a total of 20 μL was given separately as two 10-μL doses with 10–15 min between doses), the majority of FITC-labeled liposomes were recovered in mouse nasal washes 4 h later; whereas FITC-labeled liposomes were not detected in the lung washes (39). In future studies, FITC-labeled PA protein, admixed with pI:C, will be dosed nasally into mice to track PA proteins that reach the lungs.

In the present study, with a nasal pI:C dose as high as 40 μg, no gross inflammatory, allergic, or toxic effect was observed in mice. Ichinohe *et al.* (31) also reported that when pI:C was nasally given to mice daily for 9 days (25 μg/day), the body weight of the mice did not significantly change (31). Histopathological examination likewise did not reveal any pathological change in the nasal areas of mice dosed with pI:C (31). In our studies, an overall 40% fatality was observed when mice were nasally dosed with CT (1 μg/mouse once a week for 2 or 3 weeks); whereas no fatality was

observed in other groups of mice that were not treated or were dosed with vaccines that did not contain CT. All these findings suggest that pI:C is a strong and safe nasal mucosal adjuvant. Thus, more investigations on the nasal mucosal adjuvanticity of pI:C are warranted. Finally, it needs to be pointed out that in a previous clinical trial using as high as 75 mg of pI:C/m^2 on day 0 and then daily from day 7 to a maximum of 35 days, pI:C induced a number of side effects, including renal failure and hypersensitivity reactions in some patients (40), which prompted the development of a much safer, modified form of pI:C, poly(I:C_{12}U), by introducing unpaired uracil and quinine bases in the pI:C (41). Recently, it was shown that poly(I:C_{12}U) was as effective as pI:C in inducing the maturation of human monocyte derived DCs *in vitro* (42). In future studies, the poly(I:C_{12}U) may be used to replace pI:C, if needed.

In conclusion, we reported that nasal immunization of mice with anthrax PA protein with pI:C as an adjuvant induced strong PA-specific immune responses with anthrax lethal toxin neutralizing activity both in the systemic compartment and in the BALs. The strong immune response was likely to be due to the effect of pI:C on the immune system, such as the DCs, through dsRNA/TLR3 signaling. This nasal pI:C-adjuvanted, rPA-based vaccine has the potential to be developed into a nasal anthrax vaccine to prevent against an inhalation anthrax infection.

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