Effective Mucosal Immunity to Anthrax: Neutralizing Antibodies and Th Cell Responses Following Nasal Immunization with Protective Antigen

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Effective Mucosal Immunity to Anthrax: Neutralizing Antibodies and Th Cell Responses Following Nasal Immunization with Protective Antigen

Prosper N. Boyaka, Angela Tafaro, Romy Fischer, Stephen H. Leppla, Kohtaro Fujihashi, and Jerry R. McGhee

Mucosal, but not parenteral, immunization induces immune responses in both systemic and secretory immune compartments. Thus, despite the reports that Abs to the protective Ag of anthrax (PA) have both anti-toxin and anti-spore activities, a vaccine administered parenterally, such as the aluminum-adsorbed anthrax vaccine, will most likely not induce the needed mucosal immunity to efficiently protect the initial site of infection with inhaled anthrax spores. We therefore took a nasal anthrax vaccine approach to attempt to induce protective immunity both at mucosal surfaces and in the peripheral immune compartment. Mice nasally immunized with recombinant PA (rPA) and cholera toxin (CT) as mucosal adjuvant developed high plasma PA-specific IgG Ab responses. Plasma IgA Abs as well as secretory IgA anti-PA Abs in saliva, nasal washes, and fecal extracts were also induced when a higher dose of rPA was used. The anti-PA IgG subclass responses to nasal rPA plus CT consisted of IgG1 and IgG2b Abs. A more balanced profile of IgG subclasses with IgG1, IgG2a, and IgG2b Abs was seen when rPA was given with a CpG oligodeoxynucleotide as adjuvant, suggesting a role for the adjuvants in the nasal rPA-induced immunity. The PA-specific CD4+ T cells from mice nasally immunized with rPA and CT as adjuvant secreted low levels of CD4+ Th1-type cytokines in vitro, but exhibited elevated IL-4, IL-5, IL-6, and IL-10 responses. The functional significance of the anti-PA Ab responses was established in an in vitro macrophage toxicity assay in which both plasma and mucosal secretions neutralized the lethal effects of Bacillus anthracis toxin.


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often termed the edema toxin, while the PA$_r$ (LF)$_n$ molecule is named lethal toxin (LeTx).

The B subcomponent PA is now well accepted as the major immunogen for the induction of protective immunity against anthrax. Thus, the current AVA is a PA-based vaccine consisting of aluminum hydroxide-adsorbed culture filtrates from the toxigenic nonencapsulated B. anthracis strain V770-NPI-R. This AVA requires six immunizations over 18 mo, with yearly boosting (18). A number of studies have shown a major role for neutralizing anti-PA Abs for protection against anthrax (4, 5, 19–21). Further, anti-PA Abs have been shown to recognize spore-associated proteins, to stimulate spore uptake by M$_d$$_b$ and to interfere with germination of spores in vitro (7, 22). We hypothesized that a nasal vaccine consisting of recombinant PA and the well-described mucosal adjuvant cholera toxin (CT) would induce anti-PA immunity in both peripheral blood and mucosal secretions. We also characterized the nature of CD4$^+$ Th cells and cytokine responses to determine whether potential interactions of PA with its receptor would influence the type of immune response induced by this nasal recombinant PA (rPA)-based vaccine.

Materials and Methods

Mice

Female C57Bl/6 mice, 6–7 wk of age, were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens as determined by plasma Ab screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water ad libitum and were between 9 and 12 wk of age when used for these experiments. All mouse studies have been performed in accordance with both National Institutes of Health and the University of Alabama Institutional guidelines to avoid pain and distress.

Nasal immunization

Mice were nasally immunized, at weekly intervals for 3 consecutive wk, with various doses of B. anthracis rPA together with 1 $\mu$g of native cholera toxin (CT; List Biological Laboratories, Campbell, CA) as adjuvant. The rPA was produced as previously described from a recombinant strain of B. anthracis (23). Briefly, the nonsporogenic, avirulent B. anthracis strain BH445 expressing the PA gene was grown at pH 7.5 in a fermentor using M9262 anesthetized and given a total volume of 10$^3$ ml placed into each nostril. Blood and mucosal secretions (fecal extracts and vaginal washes) were collected on day 21 when mice were sacrificed.

ELISPOT assay for detection of Ab-forming cells (AFCs)

For evaluation of PA-specific AFCs in mucosal and peripheral lymphoid tissues, an IgG-specific ELISPOT assay was previously described (28, 29, 31). Briefly, dispersed cells were resuspended in RPMI 1640 medium (Cellgro Mediatech, Washington, D.C.) containing 10% FCS, 15 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (complete medium). Different dilutions of cell suspensions were then added to 96-well, nitrocellulose-based plates (Millipore, Bedford, MA) coated with 2.5 $\mu$g/ml of PA and incubated for 6 h at 37°C in a 5% CO$_2$ atmosphere. The number of PA-specific AFCs was detected with peroxidase-labeled anti-mouse $\mu$, $\gamma$, or $\alpha$-chain Abs (Southern Biotechnol- ogy Associates). Spots were visualized by adding the chromogenic substrate, 3-aminoo-9-ethylcarbazole (Moss, Pasadena, MD) and counted with the aid of a dissecting microscope (SZH Zoom Stereo Microscope System; Olympus, Lake Success, NY).

Ag-specific CD4$^+$ positive T cell responses

Single-cell suspensions from spleen and cervical lymph nodes (CLN) were obtained as previously described (30–32). The cells were first added to a nylon wool column (Polysciences, Warrington, PA) and incubated for 1 h at 37°C to obtain an enriched T cell fraction. The nonadherent, T cell-enriched population was incubated with biotinylated anti-CD4 mAb (clone GK1.5), followed by streptavidin-coupled microbeads (Miltenyi Biotec, Sunnyvale, CA). The CD4$^+$ T cells in the CD4$^+$ fraction were sorted at $>$95% purity by positive sorting by MACS (Miltenyi Biotec). Purified CD4$^+$ T cells from individual spleens or from pooled lymph node cells of 5–10 mice were cultured at a density of 4 $\times$ 10$^5$ cells/ml and stimulated with various concentrations of rPA in the presence of T cell-depleted, irradiated (3000 rad) splenic feeder cells (8 $\times$ 10$^5$ cells/ml) and IL-2 (10 U/ml; BD PharMingen) in complete RPMI 1640 medium. To measure CD4$^+$ T cell proliferation, 0.5 $\mu$Ci of tritiated thymidine ($^3$H)dU (DuPont/NEN, Boston, MA) was added to individual culture wells 4 days later. Eighteen hours after addition of $^3$HdTdR, the cells were harvested onto glass microfilter paper (Whatman, Clifton, NJ), and $^3$HdTdR incorporation was determined by liquid scintillation counting.

Analysis of PA-induced cytokine responses

Culture supernatants from CD4$^+$ T cells restimulated in vitro with rPA were collected after 5 days of incubation and subjected to cytokine-specific ELISA as described previously (29–31, 33). The assays were performed on Nunc MaxiSorp Immunoplates (Nunc, Naperville, IL) coated with anti-mouse IFN-γ, (clone R4-6A2), IL-2 (clone JES6-1A12), IL-4 (clone BV4D14-D11), IL-5 (clone TRFK5), IL-6 (clone MP5-20F3), or IL-10 (clone JES5-2A3) mAbs (BD PharMingen) in 0.1 M sodium bicarbonate buffer (pH 9.5). After blocking, cytokine standards and serial dilutions of culture supernatants were added in duplicate. The plates were washed and incubated with secondary biotinylated anti-mouse IFN-γ, (clone XMG-1.2), IL-2 (clone JES6-5H4), IL-4 (clone BV6D-24G2), IL-5 (clone TRFK4), IL-6 (clone MP5-32C11), or IL-10 (clone JES5-16E3) mAbs (BD PharMingen), followed by peroxidase-labeled goat anti-biotin Ab (Vector Laboratories, Burlingame, CA). The color was developed with ABTS as described above. Standard curves were generated using mouse rIFN-γ, IL-5, IL-6, and IL-10 (R&D Systems, Minneapolis, MN); rIL-2 (BD PharMingen); and rIL-4 (Endogen, Boston, MA). The ELISAs were capable of detecting 5 pg/ml IL-2, IL-4, and IL-5; 15 pg/ml IFN-γ; and 20 pg/ml IL-6 and IL-10.

Mbo toxicity assay to assess neutralizing Abs

The protective effects of immune plasma and mucosal secretions from mice nasally immunized with rPA plus CT were investigated by analyzing their capacity to protect the J774 M$^+$ cell line from LeTx (34, 35). Briefly, J774 M$^+$ were added to 96-well, flat-bottom wells (5 $\times$ 10$^4$ M$^+$/well) and incubated at 37°C in 5% CO$_2$ in air. After 12 h of incubation, LeTx (i.e., 400 ng/ml rPA plus 40 ng/ml LFa) was added to cultures and incubated for an additional 12 h. Viable M$^+$ were evaluated by colorimetric assay after addition of MTT (Sigma-Aldrich) (36). MTT was used at a concentration of 5 mg/ml, and a volume of 20 $\mu$l (100 $\mu$g/ml) was added to individual wells. The plates were then incubated for an additional 2 h. Serial 2-fold dilutions of plasma or mucosal secretions were added to J774 M$^+$ cultures together with LeTx to assess neutralization titers. The well-characterized neutralizing mAbs, 14B7 and 1G3, were used as positive controls (4, 37, 38).

Statistics

The results shown are reported as the mean ± 1 SE. Statistical significance ($p < 0.05$) was determined by Student’s $t$ test and the Mann-Whitney $U$
Results

Plasma anti-PA Ab isotype responses of mice nasally immunized with a rPA vaccine

We first determined the optimal dose of rPA for nasal immunization of mice. Unlike many protein vaccines, the PA of B. anthracis possesses a receptor expressed by mammalian cells that may influence the type of immune response induced (14). Nasal delivery of 10, 25, or 40 μg of rPA induced low plasma anti-PA Ab responses (reciprocal log₂ titers of 10, 11, and 14) 1 wk after the last immunization. Co-administration of rPA and CT as mucosal adjuvant promoted strong anti-PA Ab responses (Fig. 1). In fact, mice nasally immunized with CT and 10 μg of rPA displayed high levels of anti-PA IgG Ab responses, and the Ab levels were higher when the rPA dose was increased to 25 or 40 μg (Fig. 1). Plasma IgA Ab responses were not seen after nasal immunization with CT and 10 μg of rPA, and only minimal IgA responses were induced in mice that received the 25-μg rPA dose (Fig. 1). However, high plasma IgA Ab responses were seen when the rPA dose was increased to 40 μg. In terms of IgE Ab responses, no significant levels of PA-specific plasma IgE Abs were seen in mice given CT and 10 μg of rPA; however, PA-specific IgE Abs were detected in mice that received the 25- and 40-μg rPA doses (Table I).

Nasal immunization induces secretory-IgA (S-IgA) anti-PA Abs

Both nasal and oral immunization are well established to be the most reliable strategy for inducing mucosal immunity for optimal protection of mucosal surfaces (8). Significant S-IgA anti-PA Abs were seen in mucosal secretions of mice immunized with 40 μg of rPA (Fig. 3A). These results were further confirmed at the single-cell level. Thus, the frequencies of PA-specific AFCs in CLN, submandibular glands (SMG), and spleen of mice given nasal rPA (40 μg) plus CT as mucosal adjuvant were examined by Ag-specific ELISPOT assay. High numbers of PA-specific IgG and IgA AFCs were seen in CLN (Fig. 3B); however, markedly increased numbers of PA-specific IgA AFCs were noted in the SMG of mice given the nasal rPA vaccine (Fig. 3B). Different isotypes of anti-PA AFCs were detected in the spleen, where predominant IgG AFCs and only minimal IgM and IgA AFCs were present (Fig. 3B). Taken together, these results show that higher numbers of IgA AFCs are induced in mucosal effector tissues compared with systemic sites. In addition, CpG promotes mucosal S-IgA anti-PA Ab responses with mean reciprocal log₂ titers of 6 and 7 in fecal extracts and vaginal washes, respectively.

PA-specific CD4⁺ T cell responses in mice nasally immunized with rPA plus CT as adjuvant

To date, studies of immune responses to anthrax have focused on Ag responses to PA or other toxin components, and essentially no studies have assessed Ag-specific T cell responses. Purified splenic CD4⁺ T cells from mice given nasal rPA (40 μg) and CT as adjuvant exhibited significant proliferative responses to in vitro restimulation with rPA, and the optimal response was induced by 12.5 μg/ml PA (Fig. 4A). Further, similar proliferative responses were noted with CLN and splenic CD4⁺ T cells stimulated in vitro with this dose of rPA (Fig. 4B). The supernatants were collected after 5 days of culture and subjected to cytokine-specific ELISA. Control, unstimulated cells secreted low levels of IL-2 and IFN-γ, which were not significantly different from those of the Th2-type cytokines IL-6 and IL-10 (Fig. 5). On the other hand, no significant levels of IL-4 or IL-5 were noted in these control, unstimulated cultures (Fig. 5). In vitro restimulation of CLN or splenic CD4⁺ T cells with rPA promoted only minimal levels of IL-2 and IFN-γ synthesis, which did not differ from levels seen in culture supernatants of control, unstimulated T cells (Fig. 5). On the other hand, elevated Th2-type cytokine responses (IL-4, IL-5, IL-6, and IL-10) were noted after in vitro restimulation of CD4⁺ T cell cultures from both CLN and spleen (Fig. 5). Thus, as previously reported with other protein Ags (26), the PA-specific CD4⁺ T cell responses induced by the mucosal adjuvant CT were predominantly of the Th2 type. In other studies, mice nasally immunized with rPA

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Plasma anti-PA Ab isotype responses following nasal immunization with increasing doses of rPA and CT as mucosal adjuvant. Mice were immunized three times at weekly intervals with 10 μg (□), 25 μg (△), or 40 μg (■) of rPA plus 1 μg of CT as adjuvant. Plasma levels of anti-PA Abs were determined 1 wk after the last immunization (day 21). The results are expressed as the reciprocal log₂ titers ± 1 SE from three separate experiments and five mice per group per experiment.

<table>
<thead>
<tr>
<th>Nasal Immunization with</th>
<th>PA-Specific IgE Abs (reciprocal log₂ titers)</th>
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<tr>
<td>10 μg of rPA only</td>
<td>&lt;5⁺</td>
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<tr>
<td>25 μg of rPA only</td>
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<tr>
<td>40 μg of rPA only</td>
<td>&lt;5</td>
</tr>
<tr>
<td>10 μg of rPA plus CT</td>
<td>6.0 ± 1.5</td>
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<tr>
<td>25 μg of rPA plus CT</td>
<td>8.2 ± 1.7</td>
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<tr>
<td>40 μg of rPA plus CT</td>
<td>13.6 ± 0.8</td>
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*Groups of five mice were nasally immunized three times at weekly intervals (days 0, 7, and 14) with the indicated doses of rPA alone or together with 1 μg of CT as adjuvant. The PA-specific plasma IgE Ab levels were determined on day 14 as described in Materials and Methods. Results are from one experiment and are representative of three separate experiments.

*Limit of detection for Ag-specific IgE Abs.
plus CpG ODN as mucosal adjuvant exhibited PA-specific CD4+ T cell responses of a Th1-type (data not shown).

Neutralizing PA-specific Abs in plasma and external secretions

To determine whether anti-PA Ab responses were protective, both plasma and mucosal secretions were assessed in J774 Mφ cultures treated with a lethal dose of *B. anthracis* LeTx. The analysis of plasma samples collected on days 7, 14, and 21 showed neutralizing Abs in mice given 40 μg of rPA on day 7 (Fig. 6). On the other hand, booster doses of vaccine were needed to induce neutralizing Abs at the lower doses (i.e., 10 or 25 μg) of rPA. Thus, neutralizing Abs were measured in the plasma collected on day 14 from mice given 10 or 25 μg of rPA plus CT as adjuvant. The levels of plasma neutralizing Abs in these mice increased by day 21, although titers remained lower than those seen in mice immunized with 40 μg of rPA (Fig. 6).

We also analyzed the kinetics of neutralizing Ab activity in mucosal secretions including fecal extracts and vaginal washes of mice nasally immunized with 40 μg of rPA plus CT. No neutralizing Abs were detected in vaginal washes or fecal extracts after a single nasal immunization. On day 14 the majority of mice showed neutralizing Ab activity in vaginal washes, and the titers were further enhanced by day 21, 1 wk after the mice had received the second vaccine boost (Fig. 7A). The kinetics of neutralizing Ab activity were slightly different in fecal extracts, since only two of 10 mice displayed neutralizing Ab activity on day 14. More mice displayed neutralizing Abs in fecal extracts on day 21, although the responses remained generally lower than those seen in vaginal

![Fig. 2](http://www.jimmunol.org/)

![Fig. 3](http://www.jimmunol.org/)
washes. Finally, all mice given 40 μg of rPA plus CT showed neutralizing Ab activity in the nasal washes and saliva 1 wk after the last immunization (day 21; Fig. 7B).

**Discussion**

Optimal protection of the general population against either inhalational or gastrointestinal anthrax requires two layers of defense, provided by the mucosal and the systemic immune systems. In this regard, Abs to anthrax PA have now been reported to display both anti-toxin and anti-spore activities (7, 22). The systemic immunity can be achieved by intradermal or i.m. injection of anthrax vaccines, such as the current AVA. However, compartmentalization of the mucosal and systemic immune systems makes it almost certain that injected vaccines will fail to induce protective immunity at mucosal surfaces where initial spore invasion occurs (8, 9, 30, 39).

High numbers of *B. anthracis* spores were found in the nares of rhesus macaques after aerosol exposure (41). Others have shown that large anthrax spores (>5 μm) are deposited in the upper respiratory tract (i.e., pharynx, larynx, and trachea), while smaller spores (<5 μm) are transported into lymph nodes of the lower respiratory tract (42). Of course, anthrax toxin released after germination of spores plays a major role in this disease (10, 11).

Interestingly, spores of *B. anthracis* were shown to optimally germinate at 22°C (43), a temperature more consistent with this occurrence in the upper respiratory tract. The autopsies of two subjects from the 1979 outbreak of anthrax in Russia (44, 45) and studies in rhesus macaques exposed to lethal doses of anthrax spores indicated hemorrhagic and necrotizing pneumonia consistent with anthrax toxin release in the upper respiratory tract (46). In addition, nausea and vomiting observed during the recent cases of bioterrorism-related anthrax in the U.S. suggest that both the upper respiratory and gastrointestinal tracts may be early targets of the anthrax toxin after inhalation of anthrax spores (1). The results reported here clearly show that protective anti-anthrax immunity can be achieved in both mucosal and systemic lymphoid compartments by nasal immunization with rPA and an appropriate mucosal adjuvant.

Our results clearly show that only limited plasma anti-PA Ab responses can be achieved when rPA is administered alone by the nasal route. However, high levels of IgG and IgA Ab responses are
generally promotes CD4 of host protection. It has been shown that CT as mucosal adjuvant fixing IgG1 and IgG2b) are involved in distinct mechanisms ment- fixes IgG2a and IgG3 Abs vs the noncomple- mentary responses (51), and different IgG subclasses (i.e., the complement-fixing IgG2a and IgG3 Abs vs the noncomplement-fixing IgG1 and IgG2b) are involved in distinct mechanisms of host protection. It has been shown that CT as mucosal adjuvant generally promotes CD4+ Th2-associated IgG subclasses with IgG1 and IgG2b and no or minimal IgG2a Ab responses (23, 52, 53). The induction of PA-specific IgG1 and IgG2b AB responses by nasal PA plus CT suggests that PA only acted as a protein Ag and did not influence the adjuvanticity of CT. This idea was further investigated by analyzing the profile of IgG subclass after nasal immunization with CpG ODN 1826 as adjuvant. In contrast to mice given rPA plus CT, those that received CpG ODN developed high IgG2a Ab responses, which were of the same magnitude as the IgG1 and IgG2b responses.

To date, no studies have addressed mucosal IgA Ab responses to the tripartite anthrax toxin or to spores, perhaps because of the dogma that anthrax is a systemic disease resulting from the release of anthrax toxin in peripheral lymph nodes and in the general circulation (10, 11). As indicated above, there is compelling evidence that the upper respiratory tract and other mucosal tissues are affected by inhalational anthrax (1, 44–46). Further, the report that rabbit and monkey anti-PA sera stimulate spore uptake and interfere with germination (7) argues for a potentially protective role of anti-PA Abs in mucosal tissues and their secretions. Here we show that nasal immunization with an optimal rPA dose (i.e., 40 μg) plus CT promotes S-IgA anti-PA Abs in saliva and nasal washes as well as in mucosal secretions of distant mucosal sites (i.e., fecal extracts and vaginal washes). While the episodes of nausea and vomiting in victims of the recent bioterrorism-related inhalational anthrax in the U.S. could result from the systemic effects of anthrax toxin (1), one cannot exclude the possibility that these symptoms were the direct effect of anthrax toxin on mucosal tissue cells. Our results show that potential sites protected by nasal rPA vaccines could include the nasopharyngeal-associated lymphoreticular tissues, the draining lymph nodes, the lower respiratory tract, and the gastrointestinal tract as well as the systemic compartment.

seen in plasma when an optimal dose of rPA is nasally coadministered with CT as adjuvant. One could hypothesize that the presence of PA receptor (ATR) on virtually all mammalian cells (14, 47) would increase its immunogenicity. Our findings suggest that, in fact, an effective adjuvant is needed for promoting immunity to mucosally delivered PA. In this regard, anti-PA Ab responses were reported in the plasma of mice orally immunized with a Salmonella vector expressing PA (48), while no Ab response was seen after oral or nasal immunization with a Lactobacillus vector expressing PA (49). More recently, others reported the induction of anti-PA Ab responses in mice by nasal immunization with PA added to a mixture of soya phosphatidyl choline/cholate/ethanol (50). Our results obtained using the well-described mucosal adjuvant CT provide new evidence that systemic anti-PA IgG Ab responses can be induced by nasal immunization with relatively low amounts of rPA (10 or 25 μg) given with an mucosal appropriate mucosal adjuvant. However, higher doses of nasal PA were required for the induction of systemic and mucosal IgA Ab responses. Doses higher that 40 μg of rPA did not result in significantly higher responses (data not shown).

It was also important to examine the nature of IgG subclass Ab responses induced by the nasal rPA plus CT vaccine. Indeed, the pattern of IgG subclass response is known to mirror Th cell-derived cytokine responses (51), and different IgG subclasses (i.e., the complement-fixing IgG2a and IgG3 Abs vs the noncomplement-fixing IgG1 and IgG2b) are involved in distinct mechanisms of host protection. It has been shown that CT as mucosal adjuvant generally promotes CD4+ Th2-associated IgG subclasses with IgG1 and IgG2b and no or minimal IgG2a Ab responses (23, 52, 53). The induction of PA-specific IgG1 and IgG2b AB responses by nasal PA plus CT suggests that PA only acted as a protein Ag and did not influence the adjuvanticity of CT. This idea was further investigated by analyzing the profile of IgG subclass after nasal immunization with CpG ODN 1826 as adjuvant. In contrast to mice given rPA plus CT, those that received CpG ODN developed high IgG2a Ab responses, which were of the same magnitude as the IgG1 and IgG2b responses.
The functional significance of both plasma and mucosal anti-PA Abs was analyzed using the in vitro LeTx neutralization assay. High levels of neutralizing Abs were noted in plasma of mice given the optimal rPA dose (i.e., 40 μg) with CT as mucosal adjuvant. Thus, as previously reported in other systems (4, 5, 19–21), neutralizing anti-PA Abs can be generated in the plasma by a nasal rPA vaccine. It is worth noting that neutralizing Abs were also present, although at lower levels, in the plasma of mice immunized with suboptimal doses of rPA, which failed to induce a broad spectrum of anti-PA Ab isotype responses. However, the plasma did contain mainly IgG1 anti-PA Ab, which is the subclass in which mAbs have been reported to display high affinity for PA (37). Perhaps the most important finding in our study is that neutralizing Abs are induced in external secretions of mice mucosally immunized with rPA. Interestingly, neutralizing Abs were induced not only in saliva and nasal washes, but also in distal mucosal sites, since they were detected in fecal extracts and vaginal washes. It has been reported that anti-PA Ads also react with anthrax spores (5, 6, 22). Thus, the potential benefit of anti-PA mucosal immunity could be even greater if it turns out that anti-PA mucosal S-IgA Abs interfere with the germination of B. anthracis spores and/or favor spore uptake by phagocytic cells. These possibilities are currently being investigated.

To further characterize the nature of anti-PA immunity in mucosal and systemic compartments, we analyzed the pattern of Th1 and Th2 cytokine responses by PA-specific CD4+ T cells from mice nasally immunized with rPA plus CT. It is now well accepted that specific cytokines produced by Th cell subsets control the pattern of Ig isotype and IgG subclass Ab responses (51, 54). Elevated levels of IL-4 and Th2-type responses and only minimal IFN-γ secretion were detected in culture supernatants of rPA-stimulated CD4+ T cells isolated from mucosal or systemic lymphoid tissues. These findings are consistent with the pattern of PA-specific IgG subclass responses and the now well-described Th2 bias of immune responses induced by CT as mucosal adjuvant (26, 30, 52, 53, 55–57). Our study is the first to dissect the pattern of PA-specific Th cell responses after immunization with rPA. Our results again suggest that potential interactions between PA and its receptor on mammalian cells (14) are not the main factors that control the profile of PA-specific Th cell cytokine responses.

In summary, our results clearly indicate that nasal rPA represents an important avenue for the development of an effective anthrax vaccine that will provide a layer of protection at the mucosal site of pathogen entry in addition to protective immunity in the systemic compartment that is now currently achieved by theAVA.

Acknowledgments

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References


