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Evaluation of the immune response induced by a nasal anthrax vaccine based on the protective antigen protein in anaesthetized and non-anaesthetized mice

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Abstract

To better protect against inhalational anthrax infection, a nasal anthrax vaccine based on the protective antigen (PA) protein of *Bacillus anthracis* could be an attractive alternative to the current Anthrax-Vaccine-Adsorbed (AVA), which was licensed for cutaneous anthrax prevention. Previously, we have demonstrated that an anti-PA immune response comparable with that in mice subcutaneously immunized with PA protein adjuvanted with aluminium hydroxide was induced in both the systemic compartment and the mucosal secretions of the nose and lung of anaesthetized mice when they were nasally immunized with PA protein incorporated into previously reported LPD (Liposome–Protamine–DNA) particles. In this study, we evaluated the anti-PA immune response induced by the nasal PA/LPD particles in non-anaesthetized mice and compared it with that in anaesthetized mice. Our data showed that the anti-PA antibody response and the anthrax lethal toxin-neutralization activity induced by the nasal PA/LPD in non-anaesthetized mice was relatively weaker than that in anaesthetized mice. However, the splenocytes isolated from the nasally immunized mice, anaesthetized and non-anaesthetized, proliferated comparably after in-vitro re-stimulation. By evaluating the uptake of fluorescence-labelled LPD particles by phagocytes in the nasal and broncho-alveolar lavages of mice after the nasal administration, we concluded that the relatively weaker anti-PA immune response in the non-anaesthetized mice might be partially attributed to the reduced retention of the PA/LPD particles in the nasal cavity of the non-anaesthetized mice. Data collected in this study are expected to be useful for future anthrax nasal vaccine studies when mice are used as a model.

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Introduction

Bacillus anthracis is the aetiologic agent of anthrax. There are three clinical forms of anthrax infections, cutaneous, gastrointestinal and inhalational (Ascenzi et al 2002). Without a proper antibiotic intervention, all three forms of infection may lead to massive bacteraemia and toxaemia followed by hypotension, shock and sudden death (Dixon et al 1999; Chaudry et al 2002; Mourez et al 2002). Being the most lethal, inhalational anthrax infects the host through the respiratory tract. Anthrax is a toxin-mediated disease. Anthrax toxin contains three components, the protective antigen (PA), the lethal factor (LF) and the oedema factor (EF). While each of these components is not toxic alone, they combine to form two binary toxins, the lethal toxin (LeTx; i.e. LF + PA) and the oedema toxin (i.e. EF + PA). PA binds to the anthrax toxin receptor on the cell surface and mediates the entry of LF and EF into the cytosol of host cells (Bradley et al 2001). LF and EF are only toxic when inside cells (Ascenzi et al 2002). Thus, antibodies that neutralize PA block the transport of both LF and EF into the cytosol and prevent the course of infection.

The only licensed anthrax vaccine currently available in the USA is the AVA, an aluminium hydroxide (Alum)-adsorbed, formalin-treated culture supernatant of a toxigenic *B. anthracis* strain (Puziss et al 1963). While efficacious against cutaneous anthrax as licensed, AVA has many limitations, including occasional severe side-effects, difficulty maintaining consistency and, more importantly, a lengthy and complicated dosing schedule. Moreover, its efficacy against inhalational

anthrax infection in man has yet to be confirmed. Numerous animal studies have shown that the primary immunogen in the AVA is the PA component of the anthrax toxins (Pitt et al 1999; Welkos et al 2001), and anti-PA IgG had been shown to confer protection to animals challenged with *B. anthracis* (Turnbull et al 1986; Pitt et al 2001), demonstrating that anti-PA antibodies play an essential role in the immunity against *B. anthracis*. Thus, many recent anthrax vaccine developments have focused on the production of a subunit vaccine based on the PA protein.

A nasal PA-based anthrax vaccine could be an ideal alternative to the current AVA. It is advantageous in that a nasal anthrax vaccine will be self-applicable, making it possible to vaccinate a large population of people in case of an emergency. In addition, because anthrax spores enter hosts through the mucosal surface of the respiratory tract, it is conceivable that an optimal protection against an inhalational anthrax infection will require not only systemic, but mucosal immune responses, particularly in the respiratory mucosa. Finally, because anti-PA antibodies have been shown to recognize spore-associated proteins, to stimulate spore uptake by macrophages, and to interfere with the germination of spores in-vitro (Welkos et al 2001, 2002), anti-PA antibodies induced in the respiratory mucosa should theoretically be able to neutralize spores and thus prevent the entrance of spores into hosts. Thus it was no surprise that in rabbits nasally immunized with PA in a powder form, serum toxin neutralization antibody titres alone were not predictive of the survival of rabbits after an inhalational anthrax spore challenge (Mikszta et al 2005), which was in sharp contrast to the previous belief that serum toxin neutralization antibody titres alone were predictive of the efficacy of an anthrax vaccine (Pitt et al 2001).

The nasal mucosa is an important component of the mucosal immune system and thus is an ideal site to administer vaccines, especially when a mucosal immune response in the respiratory tract is desired (Kuper et al 1992; Brandtzaeg et al 1999; Davis 2001; Kiyono & Fukuyama 2004). The launch of Nasal Flu and FluMist into the market clearly demonstrated the feasibility of nasal immunization (Glezen 2004). Although Nasal Flu was pulled off the market due to its strong association with Bell's palsy (Mutsch et al 2004), a recent post-approval review of FluMist did not reveal any unexpected serious adverse effects (Izurieta et al 2005). In fact, there had also been a few attempts to administer PA-based anthrax vaccine formulations via the nasal route in murine models (Flick-Smith et al 2002; Gaur et al 2002; Boyaka et al 2003; Mikszta et al 2005).

Previously, we have demonstrated that anti-PA immune responses with strong anthrax LeTx-neutralization activity were induced in both the bronchoalveolar lavages (BALs) and the systemic compartment of mice nasally dosed with rPA incorporated into LPD (Liposome–Protamine–DNA) particles (Sloat & Cui 2005). The LPD particles were engineered by combining cationic liposomes and protamine-condensed DNA. Upon mixing, the components spontaneously rearrange to form a virus-like structure with the condensed DNA inside the lipid membrane (Li et al 1998). In a previous clinical trial, LPD was shown to be well-tolerated (Leone et al 2000). The antigen of interest may be incorporated into the LPD particles by simply adding it into the LPD preparation before the spontaneous rearrangement (Dileo et al 2003; Cui & Huang 2005). Similar to what has been carried out in most other studies, the rPA/LPD particles were dosed to mice under anaesthesia (Sloat & Cui 2005). However, there is evidence in the literature showing that the immune response induced in mice could vary significantly, depending on whether the mice were under anaesthesia or not (Yetter et al 1980; de Haan et al 1995; Ryan et al 1999; Davis 2001; Janakova et al 2002; Bracci et al 2005). The involvement of the lung has been proven to be responsible for the variations in the immune responses induced (Janakova et al 2002).

In this study, we evaluated the anti-PA immune response induced by our nasal rPA/LPD vaccine in mice dosed with or without prior anaesthesia. We hypothesized that the anti-PA immune responses induced would be significantly different. The information collected in this study should be relevant for future anthrax nasal vaccine developments when mice are used as a model.

Materials and Methods

Materials

Female Balb/C mice, 6–8 weeks old, 18–20 g, were purchased from Charles River Laboratories (Wilmington, MA). LF and rPA were purchased from List Biological Laboratories, Inc. (Campbell, CA, USA). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and fluorescein isothiocyanate (FITC)-labelled 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (FITC-DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol, protamine, dextrose, bovine serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMB), sulfuric acid and the MTT kit were purchased from Sigma-Aldrich (St Louis, MO, USA). Plasmid DNA (pNGVL), which does not encode any antigen, was from the National Gene Vector Laboratory (Indianapolis, IN, USA). It was amplified and purified using an endo-free plasmid purification kit from Qiagen (Valencia, CA, USA). Aluminium hydroxide (Alum) gel (USP grade) was purchased from Spectrum Chemical and Laboratory Products (New Brunswick, NJ, USA). Polystyrene 96-well plates were purchased from Corning Costar (Corning, NY, USA). Horseradish peroxidase (HRP)-labelled anti-mouse immunoglobulins (IgG, IgG1, IgG2a, and IgA) were purchased from Southern Biotechnology Laboratories (Birmingham, AL, USA). RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA, USA).

Preparation of Liposome and LPD

LPD and liposomes were prepared as previously described (Cui & Huang 2005). Briefly, 100-nm size small unilamellar liposomes composed of DOTAP (10 mg mL^{-1}) and cholesterol (molar ratio 1:1) were prepared by the thin-film hydration method followed by membrane extrusions. The LPD was composed of DOTAP/cholesterol liposomes, protamine and pNGVL in a ratio of 9.0:0.6:1.0 (w/w/w), respectively. To prepare the LPD, 21.5 μL of liposome (10 mg mL^{-1}) and 7.5 μL of protamine (2 mg mL^{-1}) were dispersed in 75 μL of aqueous solution containing 10% dextrose. Another 75 μL of aqueous solution containing 50 μg of pNGVL with 30 μg of rPA was added drop-wise into the mixture of liposomes and protamine while gently shaking. The incorporation efficiency of the rPA in the LPD particles was estimated to be $\sim$ 80% using SDS-PAGE gel electrophoresis (Sloat & Cui 2005). In the SDS-PAGE gel, unincorporated rPA moved into the gel, while those incorporated remained in the loading well. A comparison of the intensity of the rPA band from rPA/LPD preparation with that when the same amount of pure rPA was loaded in a different well in the same gel allowed us to roughly estimate the incorporation efficiency.

Intranasal immunization

All animal studies were carried out following National Institutes of Health guidelines for animal use and care. Anaesthetized mice (n = 5, i.p., pentobarbital, 6–7 mg/100 g) were given a total volume of 25 μL (5 μg rPA/mouse) of the rPA/LPD in suspension, which was divided into two doses of 12.5 μL given 10–15 min apart. Non-anaesthetized mice (n = 5) were similarly given a total of 25 μL of rPA/LPD while fully awake, half in each nare. Additionally, as controls, mice (n = 5) were intranasally dosed with rPA alone in phosphate-buffered saline (PBS, 10 mM, pH 7.4), subcutaneously injected with rPA (5 μg/mouse) admixed with Alum (15 μg/mouse) as an adjuvant, or left untreated. Mice were dosed on days 0, 7 and 14. They were euthanized and bled by cardiac puncture on day 28. The experiment was repeated independently twice.

Collection of nasal lavage (NL) and broncho-alveolar lavage (BAL)

Twenty-eight days after the first immunization, mice were euthanized, and NLs and BALs were collected. The trachea was exposed. To obtain NLs, 0.2 mL of sterile PBS was passed through the trachea toward the nose and collected in a microfuge tube as it exited the nares. BALs were obtained by pipetting 0.4 mL of PBS towards the lungs via the trachea. The lavage was aspirated back into the pipet tip and re-injected once before the final withdrawal of the PBS fluid. Lavage samples were stored at -80°C before further use.

ELISA for anti-PA antibody measurement

Enzyme-linked immunosorbent assay (ELISA) was used to determine the anti-PA antibody levels. Briefly, EIA/RIA flat-bottom, medium-binding, polystyrene 96-well plates (Corning Costar) were coated with 100 ng of rPA protein dissolved in 100 μL of 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed once 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. Serum samples were diluted 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 serum samples were removed, and the plates were washed five times with PBS/Tween 20. HRP-labelled goat-anti-mouse immunoglobulin (IgG, IgA, IgG1, or IgG2a; 5000-fold dilution) was added as the secondary antibody into the plates, and the plates were incubated for 1 h at 37°C. The plates were again washed five times with PBS/Tween 20. The presence of bound antibody was detected following a 30-min incubation of the plates at room temperature in the presence of TMB substrate followed by the addition of sulfuric acid (0.2 M) as the stop solution. The absorbance was read at 450 nm using a SpectraMax Plate reader (Molecular Devices Inc., Sunnyvale, CA, USA). Anti-PA antibody levels in the BALs and NLs were also determined similarly.

Anthrax lethal toxin-neutralization activity (TNA) assay

TNA was determined as described elsewhere with modifications (Boyaka et al 2003). Confluent J774A.1 cells were plated (5.0×10^4 cells/well) in sterile, 96-well, clean-bottom plates and incubated at 37°C, 5% CO_2 overnight. A fresh solution (50 μL) containing LF (40 ng mL^{-1}) and rPA (400 ng mL^{-1}) was mixed with 50 μL diluted serum sample in triplicate and incubated for 2 h at 37°C. The cell culture medium was removed, and 100 μL of the serum/LeTx mixture was added to each well and incubated at 37°C, 5% CO_2 for 3 h. Cell viability was determined using an MTT test kit, with untreated cells and LeTx alone treated cells as controls.

Splenocyte proliferation assay

Single splenocyte suspension from individual spleens was prepared as previously described (Cui & Mumper 2002). Splenocytes were cultured at a density of 4×10^6 cells/mL and stimulated with either 0 or 12.5 μg mL^{-1} of rPA in complete RPMI medium for 5 days at 37°C, 5% CO_2 (Boyaka et al 2003). The final cell numbers were determined using an MTT kit. Cell proliferation ratio was reported as the ratio of the final splenocyte number after the cells were cultured in the presence of rPA (12.5 μg mL^{-1}) for 5 days over the final number of the same splenocyte preparation

after they were cultured in the absence of rPA ($0\,\mu g\,mL^{-1}$) for 5 days.

Uptake of FITC-labelled LPD particles by phagocytes in BAL and NL

Anaesthetized and non-anaesthetized mice (n = 3) were dosed as described above with FITC-labelled LPD particles. Four hours later, mice were euthanized, and their BAL and NL were collected. The uptake of FITC-LPD particles by phagocytes in the lavages was analysed using a flow cytometer (BD LSR II laser benchtop, San Jose, CA, USA). FITC-labelled LPD particles were prepared as stated above with DOTAP liposomes containing FITC-labelled DOPE (0.5%, w/w). The percentage of FITC-positive cells was determined using the undosed mice as a reference.

Statistics

Statistical analyses were completed using one-way analysis of variance followed by Fischer's protected least significant difference (PLSD) procedure. When two groups were compared, the *t*-test was used. $P \leq 0.05$ (two tail) was considered to be statistically significant.

Results

After intranasal immunization with rPA incorporated into LPD particles (rPA/LPD), the level of anti-PA IgG induced in anaesthetized mice was greater than that in non-anaesthetized mice

Anaesthetized mice intranasally immunized with the rPA/LPD had levels of anti-PA IgG similar to those in mice subcutaneously immunized with rPA adjuvanted with Alum in their sera 28 days after the first immunization (Figure 1A). The anti-PA IgG titres in the anaesthetized mice immunized with the rPA/LPD and in mice injected (s.c.) with rPA adjuvanted with Alum were comparable (Figure 1B). Anti-PA IgG was also induced in non-anaesthetized mice nasally immunized with the rPA/LPD, although the level was lower than that in anaesthetized mice (Figure 1A). The end-point titre of the anti-PA IgG in the non-anaesthetized mice was above 100.

The anti-PA response was Th2-biased

To understand the T helper (Th) cell response induced, the levels of anti-PA IgG subtypes, IgG1 and IgG2a, in the serum were also measured using ELISA. As shown in Figure 2, similarly to subcutaneous injection of rPA adjuvanted with Alum, anaesthetized mice intranasally immunized with rPA/LPD produced a high level of anti-PA IgG1, but not IgG2a, indicating that the immune response induced was Th2-biased. Although relatively weaker, the

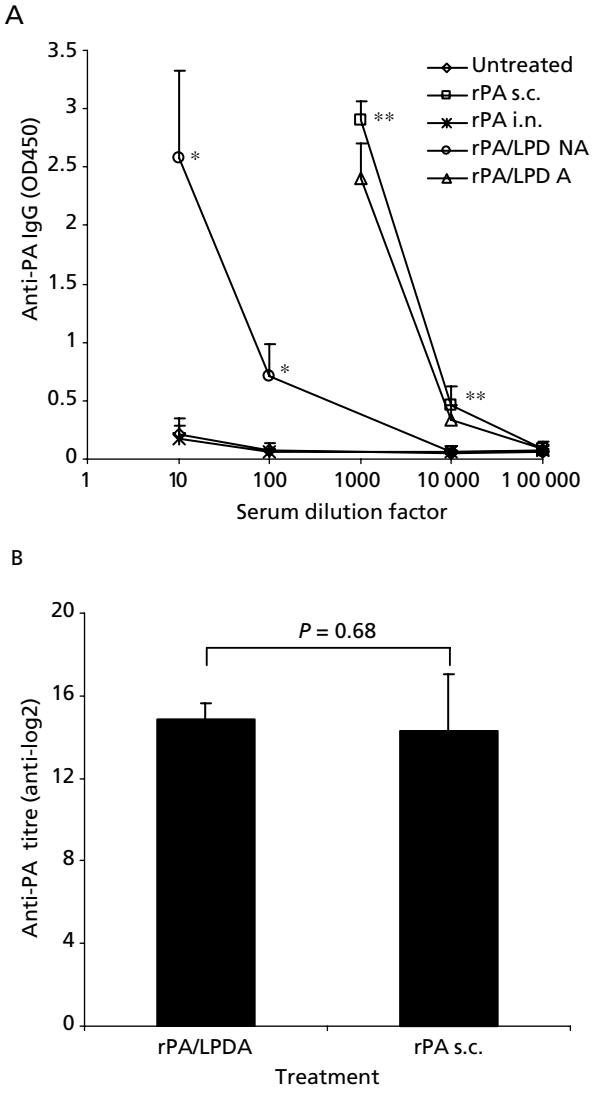

Figure 1 Serum anti-PA IgG antibody response. Anaesthetized (rPA/LPD A) and non-anaesthetized (rPA/LPD NA) Balb/C mice (n = 5) were nasally dosed with rPA (5 μg) incorporated in the LPD on days 0, 7 and 14. Other groups (n = 5) were nasally dosed (i.n.) with rPA (5 μg) alone, subcutaneously (s.c.) injected with rPA adjuvanted with Alum (15 μg/mouse), or left untreated. On day 28, mice were euthanized, and their serum samples were collected. A. The total anti-PA IgG levels in mouse sera were determined after the sera were diluted 10-fold serially. *Values for rPA/LPD (NA) were significantly different from that of the untreated and the rPA (i.n.) in the 10- and 100-fold dilutions. **At the 1000- and 10 000-fold dilutions, the values for rPA (s.c.) and rPA/LPD (A) were not significantly different from each other, but higher than that of the others. At the 100 000-fold dilution, there was no significant difference among the OD450 values from all five treatments. B. The anti-PA IgG titres in mice subcutaneously injected with rPA adjuvanted with Alum or in anaesthetized mice nasally dosed with the rPA/LPD. Serum samples were diluted 2-fold serially. The two values were not different from each other ($P = 0.68$). Data shown were mean ± s.e.m., n = 5. The experiment was repeated independently twice, and similar results were obtained. Data from one representative are shown.

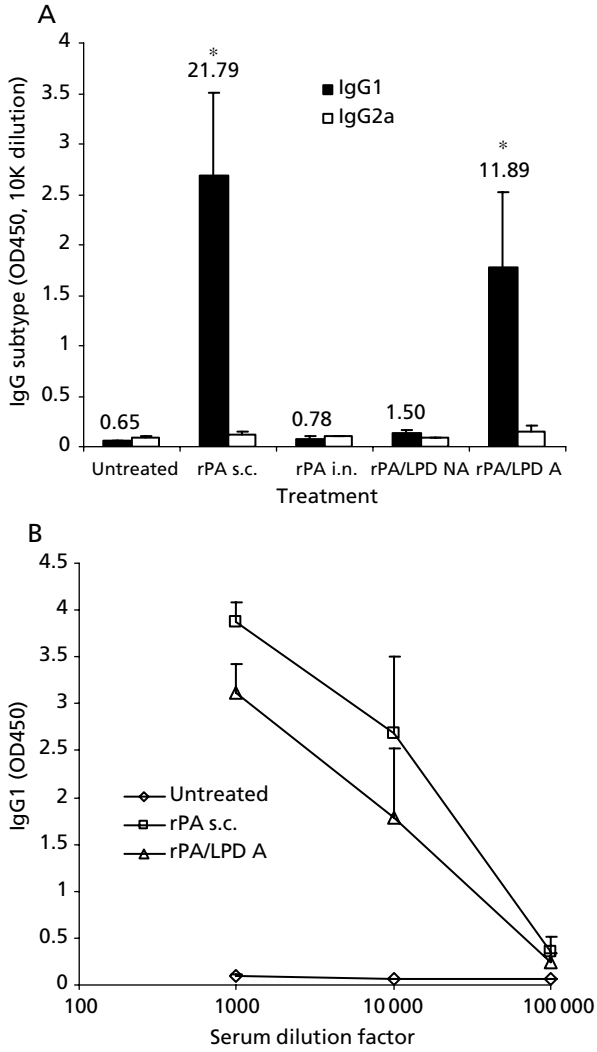

Figure 2 Serum anti-PA IgG subtypes. A. Anaesthetized (rPA/LPD A) and non-anaesthetized (rPA/LPD NA) Balb/C mice (n = 5) were intranasally dosed with rPA/LPD on days 0, 7 and 14. Other groups (n = 5) were treated (i.n.) with rPA alone, subcutaneously injected with rPA adjuvanted with Alum, or left untreated. The IgG1 and IgG2a levels in the sera on day 28 were determined using ELISA after the sera were diluted 10^4 fold. *IgG1 values for rPA (s.c.) and rPA/LPD (A) were not different from each other. Numbers above the bars were the values of the $OD450_{IgG1}/OD450_{IgG2a}$. B. The OD450 values of the anti-PA IgG1 levels in the sera of mice subcutaneously injected with rPA adjuvanted with Alum and in anaesthetized mice nasally immunized with rPA/LPD particles after the sera were diluted 1000, 10 000 and 100 000 fold. Data shown are mean ± s.d., n = 5.

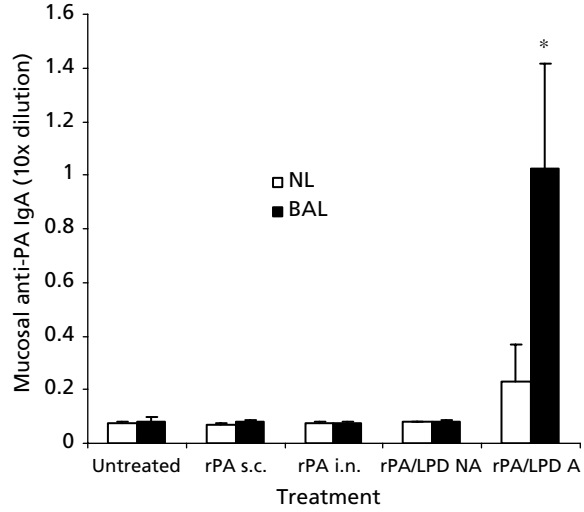

Figure 3 Anti-PA IgA in the nasal and broncho-alveolar lavages. Anaesthetized (rPA/LPD A) and non-anaesthetized (rPA/LPD NA) Balb/C mice (n = 5) were intranasally dosed with rPA/LPD on days 0, 7 and 14. Other groups (n = 5) were treated with rPA alone, subcutaneously injected with rPA adjuvanted with Alum, or left untreated. On day 28, mice were euthanized, and their NL and BAL samples were collected. The IgA level was determined using ELISA after the lavages were diluted 10 fold. *OD450 values in the NLs and BALs of the anaesthetized mice rPA/LPD (A) were significantly different from that of the others.

immune response induced by the nasal rPA/LPD in non-anaesthetized mice was also Th2-biased (Figure 2A).

Nasal rPA/LPD induced specific anti-PA IgA in the BALs and NLs of anaesthetized mice

Specific anti-PA IgA levels in the BALs and NLs were also measured using ELISA to evaluate the mucosal immune response induced. As shown in Figure 3, anti-PA IgA was detected in both the NLs and the BALs of anaesthetized mice nasally immunized with the rPA/LPD. In fact, after 100-fold dilution, the OD450 in the BAL samples of the anaesthetized mice was still more than 2-fold higher than that of the untreated control. However, anti-PA IgA was not detected in the NLs and BALs of non-anaesthetized mice when the samples were diluted 10 fold. It is possible that anti-PA IgA could be detectable at a lower dilution. As expected, no anti-PA IgA was detected in the BALs and NLs of mice subcutaneously injected with rPA adjuvanted with Alum.

Splenocytes isolated from both anaesthetized and non-anaesthetized mice immunized with nasal rPA/LPD proliferated after in-vitro stimulation with rPA

To investigate whether a proliferative immune response was induced by the nasal rPA/LPD vaccine, splenocytes isolated from immunized mice were cultured in the presence of rPA for 5 days, and the cell proliferation was measured. As shown in Figure 4, splenocytes isolated from both anaesthetized and non-anaesthetized mice nasally immunized with the rPA/LPD proliferated significantly. The proliferation of the splenocytes isolated from the non-anaesthetized mice tended to be weaker than that from anaesthetized mice, although not

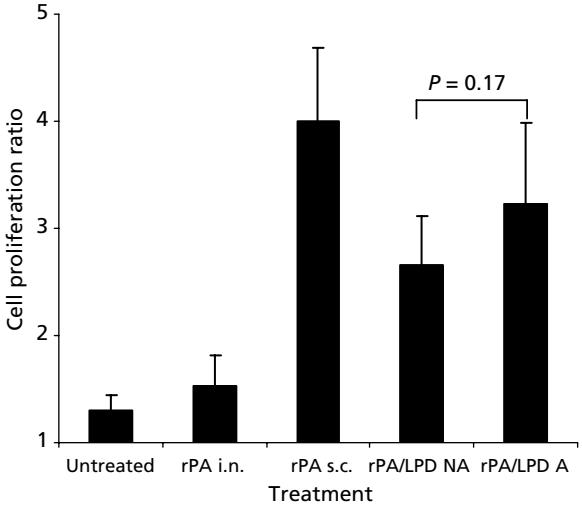

Figure 4 Proliferation of splenocytes after in vitro re-stimulation with rPA. Anaesthetized (rPA/LPD A) and non-anaesthetized (rPA/LPD NA) Balb/C mice (n = 5) were intranasally dosed with rPA/LPD on days 0, 7 and 14. Other groups (n = 5) were treated with rPA alone, subcutaneously injected with rPA adjuvanted with Alum, or left untreated. On day 28, mice were euthanized, and their spleens were harvested. Single splenocyte suspension from individual spleens was prepared. Splenocytes (4×10^6/mL) were incubated with rPA ($12.5\,\mu g\,mL^{-1}$) for 5 days. The final splenocyte number was determined using an MTT kit. The proliferation ratio was calculated by dividing the final splenocyte number after the splenocytes were cultured in the presence of rPA for 5 days by the final splenocyte number after the splenocytes were cultured in the absence of rPA for 5 days. The *P* value of 0.17 indicates that the values for rPA/LPD (NA) and rPA/LPD (A) were comparable with each other. The value for the rPA (s.c.) was not different from that of the rPA/LPD (NA) ($P = 0.13$), but was higher than that of the others.

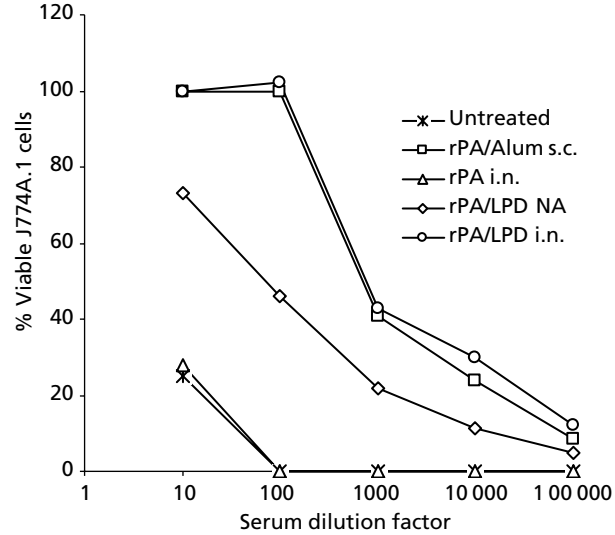

Figure 5 Intranasal rPA/LPD induced LeTx neutralization activity in both anaesthetized and non-anaesthetized mice. Balb/C mice (n = 5) were dosed as mentioned in Materials and Methods. On day 28, sera were collected. Each sample was diluted 10-fold serially and incubated with J774A.1 cells (5×10^5/mL) in the presence of anthrax LeTx ($400\,ng\,mL^{-1}$ of PA and $40\,ng\,mL^{-1}$ of LF) for 3 h. The percent of viable cells was calculated in comparison to the number of cells remained after they were treated with either LeTx alone or were left untreated. Data shown were the means from five mice. Standard deviations were not shown for a better view of the trend.

significantly due to the relatively large standard deviations observed.

Nasal immunization with rPA/LPD induced anthrax lethal toxin neutralization activity in both anaesthetized and non-anaesthetized mice

An in-vitro macrophage (J774A.1) protection assay was carried out to evaluate the protective activity of the anti-PA antibodies induced. Figure 5 shows that the LeTx neutralization activity induced in the sera of anaesthetized mice nasally dosed with the rPA/LPD was comparable with that in mice subcutaneously injected with rPA adjuvanted with Alum. The LeTx-neutralization activity detected in the serum of non-anaesthetized mice nasally dosed with rPA/LPD was weaker than that in the anaesthetized mice (Figure 5). In addition, a weak LeTx neutralization activity was also detected in the NLs and BALs of some of the mice. After a 20-fold dilution, LeTx neutralization activity was detectable in the BALs of 80% (4 out of 5) anaesthetized mice (i.e., $26 \pm 8\%$ of the J774A.1 cells were still alive when challenged with LeTx) and 3 out of 5 non-anaesthetized mice ($18 \pm 5\%$ of the J774A.1 cells were still alive). Similarly, after a 5-fold dilution, LeTx neutralization activity was detectable in the NLs of all of the anaesthetized mice ($25 \pm 3\%$ of the J774A.1 cells were still alive).

Deposition rPA-LPD particles in the nose and lung of anaesthetized and non-anaesthetized mice

To define the deposition of the nasally dosed rPA/LPD particles in the nose and lung of the mice, the uptake of rPA/LPD by phagocytes in the mucus layer of the nose and the lung was measured. A FITC-labelled rPA/LPD suspension was intranasally administered to anaesthetized and non-anaesthetized mice. Four hours later, the BALs and NLs were collected and analysed using flow cytometry. More phagocytes in the NLs of anaesthetized mice were FITC positive ($24.9 \pm 12.8\%$) than in the non-anaesthetized mice ($3.19 \pm 0.96\%$). Surprisingly, FITC-labelled rPA/LPD particles were rarely found in the BALs of both anaesthetized and non-anaesthetized mice, suggesting a very limited deposition of the rPA/LPD particles into the lung after the nasal administration.

Discussion

Previously, we have demonstrated that protective anti-PA immune responses were induced in the BALs and NLs, as

well as the systemic compartment of anaesthetized mice nasally immunized with rPA incorporated into LPD particles. In this study, we hypothesized that significant differences will exist between the anti-PA immune responses induced in anaesthetized and non-anaesthetized mice when the mice are nasally dosed with rPA/LPD. While using general anaesthesia makes it easier to intranasally dose mice, the immune responses induced in anaesthetized mice could be very different from that in the non-anaesthetized mice.

In agreement with our previous data (Sloat & Cui 2005), anaesthetized mice were found to have strong anti-PA antibody responses and LeTx neutralization activity in both the mucosal and systemic compartments (Figures 1 and 5); however, the anti-PA antibody response and LeTx neutralization activity in non-anaesthetized mice were relatively weaker (Figures 1 and 5). This is in agreement with others showing that anaesthetics, regardless of the method of delivery (e.g., intraperitoneal, intravenous, inhalational), generally led to stronger specific immune responses in anaesthetized mice (Janakova et al 2002; Bracci et al 2005). Janakova et al (2002) reported that when lightly anaesthetized mice were immunized with a meningococcal outer membrane vesicle vaccine, the median antibody concentrations were close to 10 times higher than those in non-anaesthetized mice. Similarly, Bracci et al (2005) reported that a single intranasal immunization of anaesthetized mice with haemagglutinin mixed with type I interferon (IFN) as an adjuvant induced a significant increase in serum IgG and IgA, while that induced in non-anaesthetized mice was relatively weaker. It has been shown that in fully awake mice, fluid applied to the nares was not easily inhaled, but a volume of 20 μL or more was inhaled rapidly while the mice were under anaesthesia (Yetter et al 1980). Moreover, it was demonstrated that particles administered as drops of liquid suspension into the nares of anaesthetized mice filled the lower respiratory tract 1000-fold more than in non-anaesthetized mice (Janakova et al 2002). Antigens taken up by the bronchus-associated lymphoid tissues (BALTs) in the lung was thought to contribute to the stronger immune response induced in anaesthetized mice (Neutra et al 1996; Eyles et al 1998). It was previously considered to be advantageous to have a vaccine reach the lung to achieve a total respiratory immunization in man (Yetter et al 1980; el Guink et al 1989; de Haan et al 1995). However, deeply inhaled vaccine may lead to severe pulmonary inflammation (Szarka et al 1997; Saunders et al 1999; Simecka et al 2000).

The anaesthetized mice had a strong anti-PA IgA response in both the BALs and NLs (Figure 3). Similarly, LeTx neutralization activity was detected in 5 out of 5 of the NLs (5-fold dilution) and 4 out of 5 of the BALs (20-fold dilution) of the anaesthetized mice. However, no anti-PA IgA was detected in the BALs and NLs of non-anaesthetized mice when the lavages were diluted 10 times (Figure 3). Weaker LeTx neutralization activity was detected in the BALs of some of the non-anaesthetized mice, suggesting the existence of a weak anti-PA immunity in them. Our results were consistent with a previous study using a different vaccine, where measurable levels of secretory IgA were detected in the respiratory tract of anaesthetized mice, but not in non-anaesthetized mice (Bracci et al 2005). Interestingly, Janakova et al (2002) reported that anaesthesia led to a higher level of specific antibodies in serum but not in the mucosal secretions. Janakova et al (2002) lightly anaesthetized mice by intravenous injection of propofol. The mice recovered completely 1–2 min after the anaesthetic administration (Janakova et al 2002). However, it is unlikely that this light anesthesia per-se was responsible for the difference observed. For example, de Hann et al (1995) previously showed that the mucosal immune responses induced by a nasal vaccine in heavily anaesthetized mice (i.e., pentobarbital) were significantly stronger than that in non-anaesthetized mice. Similarly, Ryan et al (1999) also showed that the specific IgA titres induced by a nasal vaccine in lightly anaesthetized mice (i.e., isofluorane) were also significantly stronger than those in non-anaesthetized mice.

To preliminarily elucidate the mechanism behind the weaker anti-PA response induced in non-anaesthetized mice by our rPA/LPD particles, we identified the location of the rPA/LPD particles by measuring the uptake of nasally administered FITC-labelled LPD particles by phagocytes in the NLs and BALs. Surprisingly, unlike what has been reported by others, no significant difference was revealed in the uptake of FITC-labelled LPD particles by the phagocytes in the BALs from anaesthetized and non-anaesthetized mice. In fact, the uptake of FITC-labelled LPD particles by phagocytes in the BALs in both anaesthetized and non-anaesthetized mice was very limited (i.e. $< 1\%$ of the phagocytes were FITC positive). Eyles et al (1998) demonstrated that a volume of 50 μL of nasal solution could result in as high as 40% of it entering the lung; however, when a smaller volume of 10 μL was dosed, it was primarily retained in the nasal airways in mice (Eyles et al 1998). In our study, a volume of about 25 μL was divided into two volumes of 12.5 μL, which were then administered 10–15 min apart, suggesting that 25 μL was still a relatively small volume. We suspect that the greater retention of FITC-LPD particles in the nasal airways of the anaesthetized mice than in the non-anaesthetized mice might contribute to the increased anti-PA IgG response and LeTx neutralization activity induced in the anaesthetized mice (Figures 1–3, 5). Finally, although it is difficult to predict to what extent the nasally administered rPA/LPD particles had travelled into the gastrointestinal tract, we believe that the contribution of the gut-associated lymphoid tissues (GALTs) in the induction of anti-PA immune responses by the rPA/LPD particles was very limited. A previous study had shown that, when influenza surface antigen haemagglutinin (HA)-entrapped liposomes were orally dosed to mice, they failed to induce any anti-HA immune responses, while the same amount of the HA-entrapped liposomes, when dosed intranasally, were able to induce strong anti-HA immune responses, both systemically and mucosally (de Haan et al 1995).

Although the anti-PA IgG level in non-anaesthetized mice was much lower than in anaesthetized mice, it was

interesting to observe that splenocytes isolated from anaesthetized and non-anaesthetized mice proliferated comparably after a five-day in-vitro re-stimulation (Figure 4). The re-stimulation step might have made the otherwise different proliferation rates comparable (Lu et al 2004). Moreover, it is important to point out that, although the anti-PA antibody response induced by the rPA/LPD in the anaesthetized mice was significantly stronger than that in the non-anaesthetized mice, the immune response remained to be Th2 biased. Thus, anaesthesia did not change the type of the anti-PA immune response induced (Figure 2A).

Finally, from this study we can clearly conclude that the immune response to rPA in anaesthetized mice nasally dosed with rPA/LPD was stronger than that in non-anaesthetized mice. Despite this, nasal rPA/LPD still holds great potential in preventing inhalational anthrax infection. It has been suggested that, unlike in mice, vaccines administered intranasally as drops may lead to strong systemic antibody responses in man (Janakova et al 2002). Although Nasal Flu had been pulled off the market due to its strong association with Bell's palsy (Mutsch et al 2004), Flumist is currently available for human use, and no unexpected serious post-approval adverse effects have been identified with this vaccine (Izurieta et al 2005). The Flumist is administered intranasally as a single 0.5 mL dose with annual boosters to account for antigenic variations in influenza strains. A sprayer is inserted into the nose, and half of the dose is administered in each nostril. We suspect that the rPA/LPD vaccine will be more effective in fully awake subjects if it is administered using a device similar to that for the administration of Flumist. Moreover, even though the nasal rPA/LPD induced higher levels of anti-PA antibodies in anaesthetized mice than in non-anaesthetized mice, it remains to be seen whether the anaesthetics would make a difference in protection against an inhalational anthrax spore challenge. Nevertheless, our future studies will be focused on improving the immune response induced by the rPA/LPD particles in non-anaesthetized mice, which might be accomplished by either increasing the dose of the rPA/LPD or by increasing the retention of the rPA/LPD particles in the nasal cavity. We are currently preparing a mucoadhesive rPA/LPD formulation, hypothesizing that it will help retain more nasally dosed rPA/LPD particles in the nasal cavity of non-anaesthetized mice, and thus, will lead to enhanced anti-PA immune responses.

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