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## Mucosal or Parenteral Administration of Microsphere-Associated *Bacillus anthracis* Protective Antigen Protects against Anthrax Infection in Mice

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Existing licensed anthrax vaccines are administered parenterally and require multiple doses to induce protective immunity. This requires trained personnel and is not the optimum route for stimulating a mucosal immune response. Microencapsulation of vaccine antigens offers a number of advantages over traditional vaccine formulations, including stability without refrigeration and the potential for utilizing less invasive routes of administration. Recombinant protective antigen (rPA), the dominant antigen for protection against anthrax infection, was encapsulated in poly-L-lactide 100-kDa microspheres. Alternatively, rPA was loosely attached to the surfaces of microspheres by lyophilization. All of the microspheric formulations were administered to A/J mice with a two-dose schedule by either the intramuscular route, the intranasal route, or a combination of these two routes, and immunogenicity and protective efficacy were assessed. An intramuscular priming immunization followed by either an intramuscular or intranasal boost gave optimum anti-rPA immunoglobulin G titers. Despite differences in rPA-specific antibody titers, all immunized mice survived an injected challenge consisting of  $10^3$  median lethal doses of *Bacillus anthracis* STI spores. Immunization with microencapsulated and microsphere-associated formulations of rPA also protected against aerosol challenge with 30 median lethal doses of STI spores. These results show that rPA can be encapsulated and surface bound to polymeric microspheres without impairing its immunogenicity and also that mucosal or parenteral administration of microspheric formulations of rPA efficiently protects mice against both injected and aerosol challenges with *B. anthracis* spores. Microspheric formulations of rPA could represent the next generation of anthrax vaccines, which could require fewer doses because they are more potent, are less reactogenic than currently available human anthrax vaccines, and could be self-administered without injection.

Anthrax, the disease caused by the spore-forming bacterium *Bacillus anthracis*, occurs in three distinct forms, cutaneous, gastrointestinal, and pulmonary, depending upon the route of infection. Cutaneous anthrax, in which the spores infect a lesion in the skin, produces a characteristic black eschar and edema at the site of infection and can be effectively treated with antibiotics. Gastrointestinal and pulmonary anthrax infections, caused by ingestion and inhalation of spores, respectively, are more difficult to diagnose and treat successfully as the symptoms of infection are not always apparent in time for antibiotic treatment to be effective (21), so vaccination is essential to protect individuals who are likely to be exposed. There are currently two licensed anthrax vaccines available for human use. The current United Kingdom-licensed anthrax vaccine is an alum-precipitated filtrate of a *B. anthracis* Sterne strain culture which is administered by the intramuscular (i.m.) route and occasionally causes some transient reactogenicity. It also has a limited duration of protection, requiring frequent boosting for continued immunity, and there is some uncertainty concerning its protective efficacy against inhaled spores of the more virulent strains of *B. anthracis* (17). The second

vaccine, licensed for use in the United States, is AVA (anthrax vaccine adsorbed), which consists mainly of protective antigen (PA) from cultures of the unencapsulated, toxin-producing strain *B. anthracis* V770-NP1-R adsorbed onto aluminum hydroxide (12). This vaccine is administered by the subcutaneous route and has similar disadvantages; it causes occasional minor reactions at the injection site and requires annual boosting to maintain immunity (5), although it provides protection against inhalational anthrax in rhesus macaques (4, 12).

*B. anthracis* produces two virulence factors, a poly-D-glutamic acid capsule (8) and a tripartite toxin composed of PA plus either lethal factor or edema factor, resulting in lethal toxin or edema toxin (18). PA is the key component in existing vaccines that protect against anthrax infection. It is an 83-kDa protein, and when it binds to the host cell membrane receptor, it is cleaved and releases a 20-kDa fragment, which exposes the lethal factor-edema factor binding site (14). The resulting 63-kDa protein forms a heptameric pore, the lethal factor or edema factor binds, and the toxin complex is internalized by receptor-mediated endocytosis (6, 20). Therefore, it is clear that inhibition of PA binding to the host cell surface should be a strategy for protection against anthrax infection.

Antibodies to PA are essential for immunity to anthrax infection (22). To protect individuals from pulmonary anthrax infection, the immune effector mechanisms at the lung surface and in the respiratory tract may also need to be primed. This

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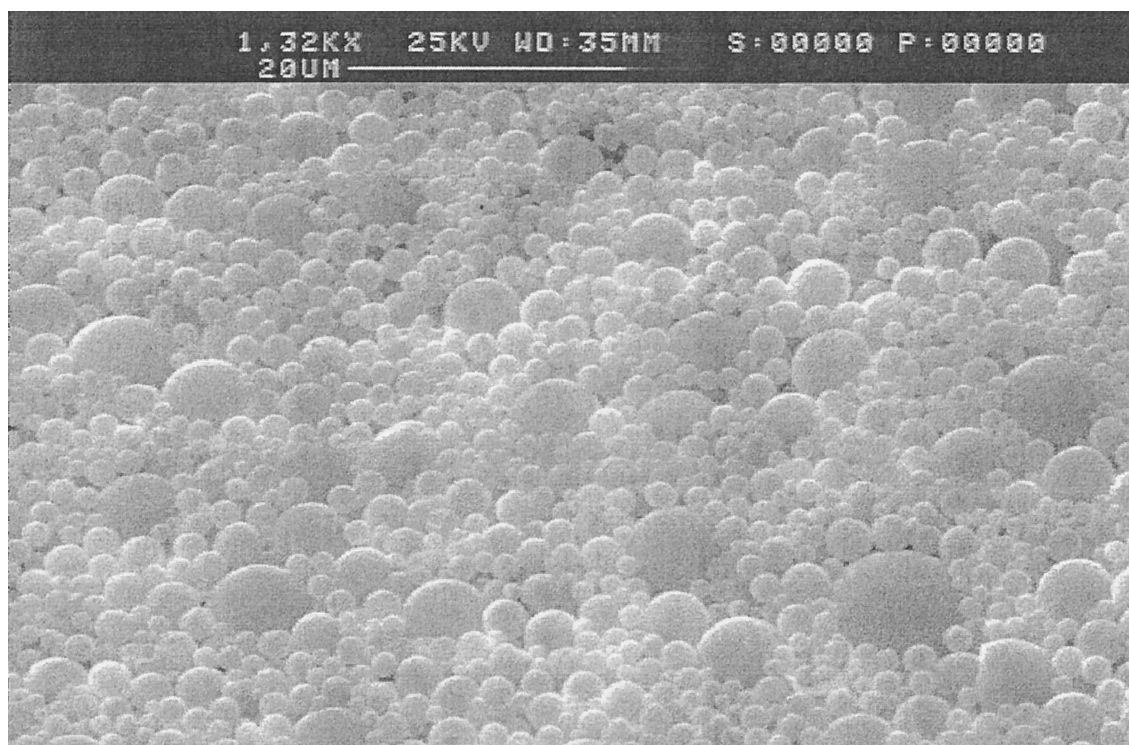


FIG. 1. Morphology of rPA-containing PLLA microspheres, as viewed by scanning electron microscopy.

can be achieved by targeting future vaccines to the mucosal immune system. Intranasal (i.n.) immunization is particularly effective at inducing mucosal immunity in the lung (2). Nasal immunization with recombinant PA (rPA) could be facilitated by encapsulation of the protein in polymeric microspheres. This has several advantages compared with more traditional vaccine formulations: it protects the protein from protease degradation; it allows sustained release of the protein in the vaccine, thus reducing the need for frequent boosting; and by making the protein a particulate antigen, it maximizes the immunogenicity of the protein.

In the present study, we investigated the optimum format for presentation of rPA in the context of a microsphere-based formulation. The immunogenicities of formulations were assessed following i.m. and i.n. administration in a two-dose immunization regimen, and the subsequent protective efficacies against both injected and aerosol challenges with anthrax STI spores in the A/J mouse model were determined.

#### MATERIALS AND METHODS

**PA.** All of the PA used in this study was rPA expressed from *Bacillus subtilis* and purified as previously described (19). Briefly, rPA was purified by ammonium sulfate fractionation of the culture supernatant, followed by ion exchange using DEAE streamline (Pharmacia), a second ion exchange using a Mono Q HR 10/10 column (Pharmacia), and finally gel filtration using a Sepharose 12 HR 10/30 column. The purified protein was determined to be more than 90% pure by scanning densitometry.

**Microencapsulation of rPA.** rPA was entrapped in polymeric microparticles composed of poly-L-lactide (PLLA) (molecular mass, 100 kDa) by using a double emulsion solvent evaporation process. Briefly, lyophilized rPA was dissolved in 0.5 ml of an aqueous solution of 2.5% (wt/vol) polyvinyl alcohol (13 to 23 kDa; 88% hydrolyzed; Aldrich, Dorset, United Kingdom). This primary aqueous phase was vigorously mixed, using a Silverson homogenizer (Silverson Machines, Bucks, United Kingdom), with an oily phase consisting of 0.25 g of PLLA

(Polysciences, Warrington, Pa.) dissolved in 5 ml of methylene chloride ( $\text{CH}_2\text{Cl}_2$ ). The resultant water-in-oil emulsion was then added, during vigorous agitation, to 75 ml of 5% (wt/vol) polyvinyl alcohol (13 to 23 kDa; 88% hydrolyzed). Following solvent evaporation overnight, hardened polymeric microparticles were harvested by centrifugation prior to lyophilization. Microspheres were characterized with respect to rPA loading, morphology, and size. Bicinchoninic acid assay (Pierce, Rockford, Ill.) analysis of 1 M NaOH-digested particles was used to ascertain the loading of rPA. Scanning electron microscopy (Cambridge Instruments, Cambs, United Kingdom) and laser diffraction measurements (Malvern Instruments, Worcs, United Kingdom) were used to investigate microsphere size and morphology. The rPA loading of the microspheres was  $2.629\% \pm 0.100\%$  (wt/wt) (entrapment efficiency, 90%). The mean particle size was  $9.5 \pm 6.8 \mu\text{m}$  (volume mean) or  $2.46 \pm 1.53 \mu\text{m}$  (number mean). The morphology of the rPA-containing microspheres is shown in Fig. 1.

**Surface modification of rPA-containing and empty microspheres.** We attempted to surface modify the antigen-containing PLLA microspheres and empty PLLA particles (prepared as described above but without rPA in the formulation) by associating additional quantities of rPA; 50-mg portions of particles were incubated (at the ambient temperature for 120 min with end-to-end shaking) in 5 ml of a 0.9% (wt/vol) solution of NaCl containing 300  $\mu\text{g}$  of rPA per ml. The particles were then treated in one of two ways: (i) they were freeze-dried as a suspension in 5 ml of incubation medium (0.9% [wt/vol] NaCl solution containing 300  $\mu\text{g}$  of rPA per ml) so that the antigen was freeze-dried onto the particles, or (ii) they were centrifuged at 21,000 rpm (Beckman J2-2 centrifuge) for 30 min, harvested, and washed three times prior to freeze-drying following suspension in 5 ml of a fresh 0.9% (wt/vol) NaCl solution. The total amounts of rPA associated (entrapped or adsorbed) with the different formulations were determined by performing a bicinchoninic acid analysis (Table 1).

**Animals.** Female 6-week-old A/J (Harlan Olac) and BALB/c (Charles River) mice were used in this study. All mice were supplied as specific-pathogen-free animals, and a microchip was embedded in each animal to aid identification. This study was carried out in strict accordance with the Animals (Scientific Procedures) Act 1986.

**Immunization and challenge studies. (i) Mucosal immune response to free rPA.** To assess the immunogenicity of rPA when it was administered i.n., each mouse in groups of five BALB/c mice was given a 5-, 10-, or 20- $\mu\text{g}$  dose of rPA either alone or with 10  $\mu\text{g}$  of cholera toxin B subunit (CTB) (Sigma Chemical Co., Dorset, United Kingdom) in 50  $\mu\text{l}$  of phosphate-buffered saline (PBS). The

TABLE 1. Adsorption of rPA onto empty and loaded 100-kDa PLLA microspheres

Formulation	Microsphere type	Modification	Location of rPA	Overall loading (% wt/wt)
1	rPA-containing spheres	Adsorb rPA	Encapsulated and adsorbed	1.501 ± 0.094
2	rPA-containing spheres	rPA weakly attached by lyophilization	Encapsulated and weakly bound	3.485 ± 0.095
2a	rPA-containing spheres	rPA weakly attached by lyophilization	Encapsulated and weakly bound	6.812 ± 0.507
3	Empty spheres	rPA adsorbed	Adsorbed	0.792 ± 0.122
4	Empty spheres	rPA weakly attached by lyophilization	Weakly bound	2.160 ± 0.160
4a	Empty spheres	rPA weakly attached by lyophilization	Weakly bound	4.957 ± 0.371
5	rPA-containing spheres	None	Encapsulated	2.629 ± 0.100
6	Empty spheres	None	NA	0

dose was administered as a mixed protein solution to the lightly anesthetized mouse (inhalational halothane) by direct instillation into the nares. After removal from the anesthetic gas, the mice revived almost immediately. The mice were immunized on days 1 and 28, and prior to booster immunization, blood samples were obtained on days 28 and 70.

**(ii) Preliminary immunogenicity and protective efficacy of microencapsulated rPA.** To assess the immunogenicity and protective efficacy of a microencapsulated formulation of rPA, groups of six female A/J mice were immunized parenterally with either microencapsulated (formulation 5 [Table 1]) or free rPA or the current United Kingdom anthrax vaccine licensed for use in humans (Centre for Applied Microbiology and Research, Wiltshire, United Kingdom). The mice received 0.4 mg of microspheres, which delivered a 10-μg dose of rPA, 10 μg of free rPA in 100 μl (total volume) of PBS (Oxoid, Basingstoke, United Kingdom), or 100 μl of human anthrax vaccine, which was equivalent to one-fifth of a human dose. All three formulations were injected i.m. by using a three-dose schedule that included boost doses of the microencapsulated and free rPA formulations given 28 and 63 days after the primary immunization and boost doses of the human anthrax vaccine given on days 28 and 42 after the primary immunization. Blood samples were taken on days 42 (prior to the boost immunization) and 84 and used for anti-PA immunoglobulin G (IgG) titer analysis performed with an enzyme-linked immunosorbent assay (ELISA), and the mice were challenged by the intraperitoneal (i.p.) route on day 105 after the primary immunization with  $1 \times 10^6$  anthrax strain STI spores, as described below.

**(iii) Screening of surface-modified microspheres for immunogenicity.** In order to enhance the immunogenicity of microencapsulated rPA formulations and therefore reduce the number of doses required to induce a protective immune response, the microspheres were modified by attaching rPA to the sphere surface either by adsorption or lyophilization, as described above. Female BALB/c mice were immunized i.m. with formulations 1 to 6 (but not formulations 2a and 4a) (Table 1). Five mice were immunized per treatment group. Each mouse received 10 μg of rPA in the encapsulated formulation in 100 μl of PBS on days 1 and 21, and mice immunized with either formulation 1 or formulation 3 received an additional 10-μg i.m. dose on day 111 after the primary immunization. The immunizing dose for each animal was divided equally between sites on the hind legs. Blood samples were taken on days 28, 42, 85, and 120 after the primary immunization.

**(iv) Protective efficacy of microencapsulated rPA.** The two most immunogenic formulations, formulations 2 and 4 (see above), were used for experiments to assess the protection against challenge provided and to ascertain if the immunization route used increased or decreased the protective efficacy. In this and subsequent experiments, female A/J mice were immunized since these mice provide a consistent mouse model for anthrax infection (24). A/J mice were immunized in groups of 10 by either the i.m. route or the i.n. route or by a combination of the two (i.m. primary immunization followed by i.n. boost immunization or vice versa) with the two formulations. Immunization by the i.m. route was carried out as described above. For i.n. immunization, sufficient microspheres to deliver a 10-μg dose of rPA per animal were suspended in 50 μl of PBS and administered to a lightly anesthetized mouse as described above. The animals were primed on day 1 and boosted on day 21. Antibody titers were monitored by taking blood samples on day 28, and the serum was separated and used for analysis by ELISA. Mice were challenged with STI spores by the i.p. route on day 55 of the immunization regimen, as described below.

In a separate experiment, A/J mice (six mice per treatment group) were immunized by using an identical regimen and either the i.m. route, the i.n. route, or a combination of the two routes with second batches of formulations 2 and 4 (formulations 2a and 4a, respectively) that were produced with a greater load of rPA per sphere (Table 1). Each mouse was immunized on days 1 and 21 with sufficient spheres to deliver a 10-μg dose of rPA, and blood samples were taken on days 28 and 45 and used for serum analysis by ELISA. The mice were

challenged by the aerosol route with STI spores on day 90 of the immunization regimen.

**Injected challenge.** For preparation of the challenge inoculum, STI spores were removed from a stock preparation, washed in sterile distilled water, and resuspended in PBS at a concentration of  $7 \times 10^8$  spores per ml. Aliquots of the spore suspension were removed, and 100-μl portions were used to challenge mice i.p. with  $7 \times 10^7$  spores per mouse (equivalent to  $10^4$  median lethal doses [MLDs] [1]). The spore suspension was diluted and used to challenge immunized mice with  $7 \times 10^6$  spores per mouse (equivalent to  $10^3$  MLDs [1]). The animals were monitored for 14 days after challenge to determine their protected status. Humane end points were strictly observed so that any animal displaying a collection of clinical signs which together indicated that it had a lethal infection was culled.

**Aerosol challenge.** Aerosol exposure was carried out by using a Henderson-type apparatus (11) and Collison spray (15). Ten-milliliter aliquots of STI spores were suspended in sterile distilled water at a concentration of  $10^9$  CFU ml<sup>-1</sup>, and 3 drops of Antifoam 289 (Sigma) was added just before each preparation was placed in the Collison spray. Mice were placed in groups of up to 20, and only their heads were exposed for 10 min to an airstream containing an aerosol of *B. anthracis* STI spores generated by the Collison spray and conditioned by the Henderson-type apparatus. The concentration of STI spores in the aerosol was determined by taking samples from the exposure chamber with an All-glass impinger (AGI-30) (16) containing sterile distilled water. Diluted impinger samples were plated onto nutrient agar to determine the number of spores per liter of aerosol. The respiratory volume of the animals was calculated by the method of Guyton (9), and the actual dose inhaled and retained in the lungs was calculated by using the data of Harper and Morton (10). Animals were closely observed for 22 days after challenge to determine their protected status.

**ELISA of serum samples to determine antibody titer to rPA.** Microtiter plates (Immulon 2; Dynex Technologies) were coated overnight at 4°C with 5 μg of rPA (expressed from *B. subtilis* [19]) per ml in PBS, except for two rows per plate which were coated with 5 μg of goat anti-mouse antibody binding fragment (FAB) (Sigma) per ml. The plates were washed with PBS containing 1% (vol/vol) Tween 20 and blocked with 5% (wt/vol) skimmed milk powder in PBS (blotto) for 2 h at 37°C. Individual serum samples, double diluted in 1% blotto, were added to the rPA-coated wells and were assayed in duplicate alongside a murine IgG standard (Sigma) added to the anti-FAB-coated wells and incubated overnight at 4°C. After washing, horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates Inc.) was added to all wells, diluted 1:2,000 in PBS, and incubated for 1 h at 37°C. The plates were washed again before addition of the substrate 2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS) (1.09 mM; Sigma). After 20 min of incubation at room temperature, the absorbance at 414 nm was determined for each well (Titertek Multiscan; ICN Flow). Standard curves were calculated by using Tittersoft software, version 3.1c. Titers were expressed either in micrograms of IgG per milliliter of serum or as the reciprocal of the end point dilution which gave an absorbance that was 0.1 U greater than the background value. Group means ± standard errors of the means were calculated.

**Statistical analysis.** The significance of the differences in anti-rPA IgG concentrations for each administration route for formulations 2, 2a, 4, and 4a was determined by analysis of variance followed by a *t* test suitable for multiple comparisons (7).

## RESULTS

### Evaluation of the immunogenicity of microencapsulated rPA compared to that of free rPA and that of the human

TABLE 2. Immune responses of A/J mice to three 10- $\mu$ g i.m. doses of rPA given either free or in a microencapsulated formulation on days 1, 28, and 63 and immune responses of A/J mice given the human anthrax vaccine (one-fifth of a human dose) on days 1, 28, and 42<sup>a</sup>

Treatment	Anti-rPA IgG titer ( $\mu$ g ml <sup>-1</sup> ) <sup>b</sup>		No. of survivors/no. challenged (%) <sup>c</sup>
	Day 42	Day 84	
Free rPA	169.31	1,044.79	6/6 (100)
Formulation 5	79.17	555.57	6/6 (100)
Anthrax vaccine	35.49	314.57	6/6 (100)
Naïve controls			0/6 (0)

<sup>a</sup> The mice were challenged on day 105 after the primary immunization.

<sup>b</sup> The values are mean anti-rPA IgG concentrations based on pooled samples taken from six mice per treatment group on days 42 and 84 after the primary immunization.

<sup>c</sup> Number of mice alive 14 days after challenge/number of mice challenged.

**anthrax vaccine.** Free rPA administered i.n. was nonimmunogenic and produced no detectable antibody response to rPA even at the highest dose (20  $\mu$ g per mouse). In comparison, when rPA was administered with the potent mucosal adjuvant CTB, anti-rPA titers of  $4.56 \pm 0.31$ ,  $4.74 \pm 0.11$ , and  $4.92 \pm 0.12$  for the mice immunized with 5, 10, and 20  $\mu$ g of rPA, respectively, were produced (titers expressed as mean log<sub>10</sub> reciprocal of the dilution  $\pm$  standard error of the mean). When administered by the i.m. route, rPA, either microencapsulated or free, was immunogenic and stimulated a protective immune response, and all immunized animals were protected against i.p. challenge with  $1 \times 10^6$  STI spores per mouse (Table 2). This showed that microencapsulating rPA did not impair its immunogenicity or protective efficacy and that it was as protective as the current human anthrax vaccine.

**Selection of the optimal formulation of microencapsulated rPA.** All the formulations screened except formulation 6 (empty spheres) were able to induce antibody to rPA. Formulations 1 (rPA encapsulated and adsorbed) and 3 (adsorbed rPA) were far less immunogenic and required three immunizing doses to achieve antibody titers similar to those obtained after two doses of formulations 2 (spheres with encapsulated and weakly bound rPA) and 4 (empty spheres with rPA weakly bound) (Table 3). Formulations 2 and 4 were found to be optimally immunogenic in the initial screening study and were used for further evaluation. When trials were performed with A/J mice, both of these formulations and the subsequently produced second batches of these formulations (formulations 2a and 4a) were able to induce substantial anti-rPA IgG titers (Fig. 2). For each formulation, significantly higher anti-rPA antibody responses were obtained with two i.m. immunizing doses than with either two i.n. immunizing doses (formulation 2,  $P = 0.02$ ; formulation 4,  $P = 0.01$ ; formulation 2a,  $P < 0.001$ ; formulation 4a,  $P = 0.007$ ) or a combination of i.n. priming followed by i.m. boosting (formulation 2,  $P = 0.07$ ; formulation 4,  $P < 0.001$ ; formulation 2a,  $P = 0.002$ ; formulation 4a,  $P = 0.02$ ). There was no significant difference between the anti-rPA IgG titers for groups immunized with formulation 2 and the anti-rPA IgG titers for groups immunized with formulation 4 for any combination of administration routes except for the group primed with formulation 2 by the i.m. route and then boosted with an i.n. dose ( $P = 0.013$ ). In contrast, immunization with

formulation 4a by any combination of administration routes except i.n. priming and boosting resulted in anti-rPA IgG titers considerably higher than the anti-rPA IgG titers for groups immunized by the same routes with formulation 2a (two i.m. doses,  $P = 0.01$ ; two i.n. doses,  $P = 0.08$ ; i.m. priming followed by i.n. boosting,  $P = 0.055$ ; i.n. priming followed by i.m. boosting,  $P = 0.004$ ). A comparison of the anti-rPA titers after immunization with formulations 2 and 2a and with formulations 4 and 4a by each combination of immunization routes showed that there was no significant difference in the titers stimulated by formulations 2 and 2a except when the formulations were administered i.m. followed by i.n. boosting ( $P = 0.04$ ), whereas the titers for mice immunized with formulation 4a were considerably higher than the titers for mice immunized with formulation 4 (two i.m. doses,  $P = 0.02$ ; i.m. priming followed by i.n. boosting,  $P = 0.05$ ; i.n. priming followed by i.m. boosting,  $P < 0.001$ ) except for the mice primed and boosted i.n., for which there was no significant difference in the anti-rPA IgG responses to the two formulations. The predominant subclass of IgG seen in samples taken from mice immunized with either formulation 2a or 4a was IgG1; there were much lower amounts of IgG2a and IgG2b and no detectable IgG3 (data not shown), indicating that there was a predominantly Th2-directed response to immunization with these formulations.

**Assessment of the protective efficacy of microencapsulated formulations of rPA.** All mice immunized with either formulation 2 or formulation 4 were fully protected against i.p. challenge with  $7 \times 10^6$  STI spores (approximately  $10^3$  MLDs). At a 1-log-higher challenge level ( $10^4$  MLDs) there was some breakthrough in protection for animals receiving formulation 2, but full protection was seen in the mice immunized with formulation 4 (Table 4). All untreated control animals rapidly succumbed to infection, and the time to death was  $2.8 \pm 0.2$  days (mean  $\pm$  standard error of the mean). The calculated retained dose of spores received by the mice challenged by the aerosol route was  $1.57 \times 10^4$  spores (equivalent to 30 MLDs by this route). Immunization by any combination of administration routes with formulation 4a provided full protection against aerosol challenge at this level. There was a single death in the mice immunized with formulation 2a when i.n. priming and i.n.

TABLE 3. Immune responses of BALB/c mice ( $n = 5$ ) to either two (formulations 2, 4, 5, and 6) or three (formulations 1 and 3) 10- $\mu$ g i.m. doses of rPA given as microencapsulated formulations on days 1 and 21 (two-dose regimen) and on days 1, 21, and 111 (three-dose regimen)

Formulation	Anti-rPA IgG titer <sup>a</sup>			
	Day 28	Day 42	Day 85	Day 120
1	ND <sup>b</sup>	1.7	2.3	$3.54 \pm 0.42$
2	$4.57 \pm 0.18$	$4.45 \pm 0.25$	$4.02 \pm 0.12$	$3.42 \pm 0.2$
3	2.6	2.0	2.0	$2.93 \pm 0.23$
4	$4.26 \pm 0.06$	$4.45 \pm 0.15$	$3.96 \pm 0.11$	$3.42 \pm 0.07$
5	$3.2 \pm 0.1$	$3.77 \pm 0.34$	$3.0 \pm 0.2$	$3.3 \pm 0.17$
6	ND	ND	ND	ND

<sup>a</sup> The titers are mean log<sub>10</sub> reciprocal serum dilutions  $\pm$  standard errors of the means; single values are titers obtained from single responding animals. The titers were determined on days 28, 42, 85, and 120 after the primary immunization.

<sup>b</sup> ND, not detectable.

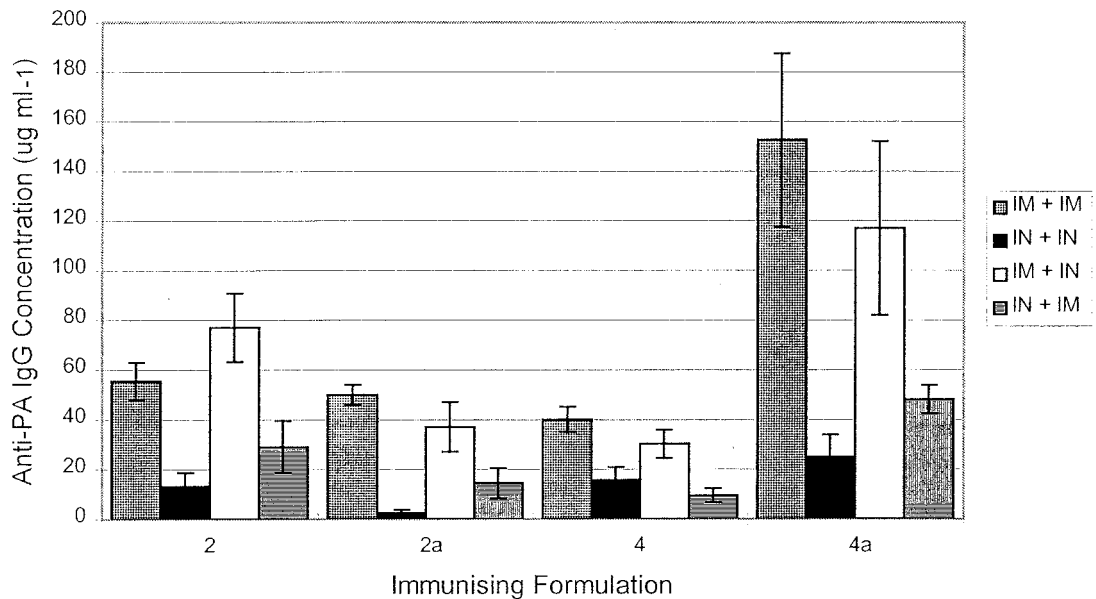


FIG. 2. Anti-rPA IgG concentrations (means  $\pm$  standard errors of the means;  $n = 5$  for formulations 2 and 4;  $n = 6$  for formulations 2a and 4a) for A/J mice immunized with sufficient formulation 2, 4, 2a, or 4a to deliver a 10- $\mu$ g dose of rPA by either the i.m. route or the i.n. route on days 1 and 21 of the immunization regimen. Samples were taken 28 days after the primary immunization.

boosting were used, but this formulation was fully protective when it was administered by the i.m. route or by a combination of i.m. and i.n. routes (Table 5). All untreated control animals died, and the time to death was  $3.4 \pm 0.5$  days (mean  $\pm$  standard error of the mean).

### DISCUSSION

For optimum protection against pulmonary anthrax infection it is probably necessary for mucosal pathways, as well as systemic immune pathways, to be primed. One way to achieve good mucosal immunity in the lungs and respiratory tract is to administer the antigen locally, in a particulate form. The presence of rPA as a particulate is critical for enhancing protective immunity when it is administered i.n. In contrast, this study showed that when delivered directly to the respiratory tract epithelium of the mouse, rPA in aqueous solution is nonimmunogenic unless it is combined with a mucosal adjuvant, such as CTB.

Immunization studies with rPA incorporated into microspheres and gel formers have been described previously (M. Kende, C. Yan, J. Corbett, G. C. Atkins, and S. W. Shalaby, Proc. Int. Symp. Controlled Rel. Bioactivated Mater. 2000, abstr. 0223), and success with microencapsulation has previously been shown in mice with other antigens that protect, for example, against *Yersinia pestis* infection (3). In this study it was found that for two strains of mice, rPA can be encapsulated in polymeric microparticles without impairment of the immunogenicity and protective efficacy of the protein. In the preliminary trial of the microencapsulated rPA formulation, the circulating anti-rPA IgG concentrations in the formulation 5-immunized mice were approximately one-half those obtained for the mice immunized with free rPA (Table 2). These titers were greater than those in the human anthrax vaccine-immunized mice, which received only one-fifth of a human

dose (approximately 0.5  $\mu$ g of PA, assuming an average vaccine PA content of 2.5  $\mu$ g per 0.5 ml [13]), 20 times less than the dose of microencapsulated rPA delivered in formulation 5. These data suggest that following i.m. injection, microencapsulated rPA is not processed and presented to the relevant elements in the immune system as effectively as free antigen. It is also feasible that exposure to sheer forces and/or organic solvents during the manufacture of this formulation may have damaged the critical antigenic epitopes of the rPA, reducing its immunogenicity. A number of studies have shown that there is no direct correlation between antibody titer to PA and protection against anthrax infection (12, 22, 25). Despite this, a certain threshold titer of antibody is most likely required for protection, and although microencapsulated rPA formulation 5 was not as immunogenic as free rPA when it was administered by the i.m. route, it induced the threshold titer necessary

TABLE 4. Survival of A/J mice immunized on two occasions (days 1 and 21) by the i.m. and i.n. routes and challenged by the i.p. route on day 55 with either  $10^3$  or  $10^4$  MLDs of STI spores

Treatment group	Routes of administration	No. of survivors/no. challenged (%)	
		$10^3$ MLDs <sup>a</sup>	$10^4$ MLDs
Formulation 2	i.m. + i.m.	5/5 (100)	4/5 (80)
Formulation 2	i.n. + i.n.	5/5 (100)	3/5 (60)
Formulation 2	i.m. + i.n.	5/5 (100)	5/5 (100)
Formulation 2	i.n. + i.m.	5/5 (100)	5/5 (100)
Formulation 4	i.m. + i.m.	5/5 (100)	5/5 (100)
Formulation 4	i.n. + i.n.	5/5 (100)	5/5 (100)
Formulation 4	i.m. + i.n.	5/5 (100)	5/5 (100)
Formulation 4	i.n. + i.m.	5/5 (100)	5/5 (100)
Naïve control		0/5 (0)	0/5 (0)

<sup>a</sup> 1 MLD, = approximately  $1 \times 10^3$  STI spores (1).

TABLE 5. Survival 22 days after aerosol challenge of A/J mice (six mice per group) immunized on days 1 and 21 by the i.m. and i.n. routes with sufficient spheres of either formulation 2a or formulation 4a to deliver a 10- $\mu$ g dose of rPA

Treatment group	Routes of administration	No. of survivors/no. challenged (%) <sup>a</sup>
Formulation 2a	i.m. + i.m.	6/6 (100)
Formulation 2a	i.n. + i.n.	5/6 (83)
Formulation 2a	i.m. + i.n.	6/6 (100)
Formulation 2a	i.n. + i.m.	6/6 (100)
Formulation 4a	i.m. + i.m.	6/6 (100)
Formulation 4a	i.n. + i.n.	6/6 (100)
Formulation 4a	i.m. + i.n.	6/6 (100)
Formulation 4a	i.n. + i.m.	6/6 (100)
Naïve control		0/8 (0)

<sup>a</sup> The animals were challenged with 30 MLDs (1 MLD = approximately 500 STI spores).

for protection and produced a level of protection comparable to that provided by the human anthrax vaccine.

Encapsulation and weak binding of antigen to biodegradable polymers can act as a continuous antigen release system, potentially reducing the number of immunizing doses required to elicit a protective immune response. This is important for patient compliance and also if there is a risk of exposure to the pathogen soon after immunization. The original encapsulated rPA formulation tested, formulation 5, was protective after three immunizing doses of rPA-containing microspheres. Compared to the number of doses recommended for the United Kingdom vaccine (four doses over a 6-month period, followed by yearly booster doses [human anthrax vaccine package insert]) or the United States vaccine (six doses over an 18-month period, followed by an annual booster dose [12]), this is a substantial improvement, but the objective was to reduce the number of doses more by further enhancing the presentation of rPA to the immune effector cells.

In addition to microspheres containing entrapped rPA, empty and rPA-containing particles with additional quantities of surface-associated rPA were prepared and tested for immunogenicity. It was anticipated that the immunogenicity of rPA might be improved by association with microparticles and that by using this method the potentially degradative elements that might be encountered during microencapsulation could be avoided. In the screening study, formulations 2 (rPA encapsulated and weakly surface bound) and 4 (rPA weakly surface bound to empty spheres) were found to be particularly promising. These formulations were used in an assessment of protective efficacy in the A/J mouse model and were found to induce protection following only two immunizing doses. In A/J mice there is a positive correlation between the immunizing dose of rPA administered and the protective antibody response stimulated, making this a suitable model for anthrax infection studies (23, 25). Due to the relatively small size of a mouse, the amount of spheres in a PBS suspension that can be administered is limited, and this also limits the immunizing dose of rPA delivered; however, with further modification of the microencapsulated formulations it may be possible to increase the deliverable rPA dose and therefore reduce the number of immunizing doses required to stimulate a protective immune response still further in order to produce a single-dose vaccine.

In this study, we also assessed how the administration route affected the immunogenicities and protective efficacies of formulations 2 and 4, which were selected based on the screening study, and the subsequently produced second batches of these formulations (formulations 2a and 4a). For all four formulations, i.m. priming followed by either i.m. or i.n. boosting resulted in the highest anti-rPA titers, and all immunization route combinations for formulations 2 and 4 protected against an injected challenge with 10<sup>3</sup> MLDs of anthrax strain STI. There was some breakthrough in protection of the mice immunized with formulation 2 (encapsulated and weakly surface-bound rPA) at the higher injected-challenge level (10<sup>4</sup> MLDs). Aerosol challenge of mice immunized with formulations 2a and 4a gave results similar to the injected-challenge results; the formulation 4a-immunized mice were fully protected against challenge with 30 MLDs of STI spores, but some breakthrough in protection was observed with the formulation 2a-immunized mice. Despite this, mice that were immunized with any of the four formulations by using a combined i.m.-i.n. schedule were fully protected at all challenge levels against both injected and aerosol challenges, which emphasized the importance of stimulating both the mucosal and systemic immune systems to obtain full protection against these types of challenges. It is perhaps noteworthy that with the exception of the i.n. primed groups, when formulations 4 and 4a were administered by using the same regimen, they did not induce the same magnitude of antibody response. It is unlikely that the performance of the different formulations could be attributed to their rPA contents, since formulation 2 contained more rPA than formulation 4 and indeed formulation 4a contained less rPA than formulation 2a. It is more likely that release of rPA and the accessibility of the rPA to the immune system are more critical criteria for effective induction of protective immunity in response to these formulations.

It has previously been demonstrated that immunization with rPA results in a Th2-directed immune response (26). This was also observed with rPA given as microspheric formulations, independent of the route of administration, showing that there is no qualitative difference in the antibody subclasses stimulated. This indicates that the protective immune mechanisms stimulated by the microsphere-associated rPA are identical to the mechanisms stimulated by the administration of rPA with other adjuvants, such as the alum used in the human anthrax vaccine, although the specific protective immune mechanism stimulated by PA immunization remains unknown.

This study demonstrated that association of rPA with biodegradable microparticles (either by entrapment or by surface association or both) is an effective approach for immunization against anthrax infection. Pharmaceutical modification of rPA in this way appears to facilitate effective delivery of the antigen to inductive sites in the mucosal immune system. We speculate that the immunogenicity of rPA may be further enhanced due to sustained release of the antigen from the polymeric carriers. It is known that microspheric material enters nasally associated lymphatic tissue, draining lymph nodes, and systemic immunological compartments following i.n. delivery. This may also contribute to the effectiveness of microsphere-associated or -entrapped rPA as a mucosal immunogen compared with the effectiveness of soluble rPA. In this study the utility of combined i.m. and i.n. immunization routes was particularly high-

lighted. This approach could be adapted for human use if an injected primary dose of microencapsulated rPA or rPA with alum as an adjuvant were followed by a nasal booster dose of microencapsulated antigen. This mixed-route regimen may be particularly appropriate because the respiratory tract epithelium is the initial site of infection following inhalation of anthrax spores, and it therefore seems logical to attempt to develop vaccination strategies that evoke appropriate localized responses to counteract the early events of pathogenesis. In this study we confirmed that robust protection against both injected and aerosol challenges with *B. anthracis* spores can be achieved by utilizing these dual immune mechanisms.

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