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Adjuvanticity and Protective Immunity Elicited by *Bordetella pertussis* Antigens Encapsulated in Poly(DL-Lactide-Co-Glycolide) Microspheres

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Purified *Bordetella pertussis* antigens, encapsulated in biodegradable poly(DL-lactide-co-glycolide) (DL-PLG) microspheres, were evaluated for their immunogenicity and ability to elicit a protective immune response against *B. pertussis* respiratory infection. Microencapsulated pertussis toxoid, filamentous hemagglutinin, and pertactin all retained their immunogenicity when administered parenterally. Intranasal immunization with a low dose (1 μg) of encapsulated filamentous hemagglutinin, pertussis toxoid, or pertactin elicited strong specific immunoglobulin G and immunoglobulin A antibody responses in respiratory secretions that were greater in magnitude than the responses elicited by the same doses of unencapsulated antigen. Intranasal immunization with as little as 1 μg of encapsulated pertussis antigen prior to infection reduced the bacterial recovery by 3 $\log_{10}$ CFU. However, intranasal immunization with the same low doses of unencapsulated antigens did not reduce infection. Intranasal administration of a combination of 1 μg of each of the microencapsulated pertussis antigens was more effective in reducing bacterial infection than administration of any single microencapsulated antigen. Intranasal administration of microencapsulated *B. pertussis* antigens elicits high levels of specific antibody coinciding with protection against infection when these microspheres are administered to the respiratory tract. These data provide evidence of the respiratory adjuvanticity of three different DL-PLG microsphere preparations, each of which contains a unique *B. pertussis* antigen.

Bordetella pertussis is a highly contagious respiratory pathogen that exhibits a distinct tropism for the cilia of the respiratory epithelium (18). *B. pertussis* remains localized to the respiratory tract during the course of disease and does not disseminate to cause bacteremia or meningitis, as do respiratory pathogens such as *Neisseria meningitidis* and *Haemophilus influenzae* (3).

While parenteral whole-cell pertussis vaccination prevents disease in young children, the population with the greatest morbidity and mortality from pertussis, immunity from this vaccine wanes in young adulthood. In contrast, convalescence from disease elicits long-lived protective immunity (13). Mucosal immunoglobulin A (IgA) antibodies to *B. pertussis* are commonly detected following disease but not following parenteral immunization (16). The restriction of this pathogen to the respiratory tract and the induction of local and long-lived immunity following disease but not following parenteral immunization suggest that the induction of a potent local respiratory immune response to antigens of *B. pertussis* may be an effective vaccine strategy.

It has previously been shown that antigens such as formalinized staphylococcal enterotoxin B and bovine serum albumin, encapsulated in biodegradable microspheres composed of poly(DL-lactide-co-glycolide) (DL-PLG), have enhanced parenteral immunogenicity and are effective mucosal immunogens (2, 6, 7). Our laboratory has used a mouse model of *B. pertussis* respiratory infection, in which the bacteria have been demonstrated to be associated with the ciliated epithelium of the murine trachea (12) and bronchial tree (25), to analyze parameters of immunity that interfere with the persistence of *B. pertussis* at the respiratory epithelium (26, 27). In this study, we utilize this infection model to characterize the immunogenicity and protective immunity of three distinct antigens purified from *B. pertussis*, each of which had been encapsulated in DL-PLG microspheres. We demonstrate that respiratory administration of each of these microencapsulated antigens, filamentous hemagglutinin (FHA), genetically inactivated pertussis toxoid, and pertactin, elicits a vigorous protective immune response coincident with high levels of specific antibodies in the serum and respiratory secretions, responses not observed with the same doses of soluble pertussis antigens.

MATERIALS AND METHODS

Mice. BALB/cAnNcR (referred to below as BALB/c) mice were obtained from the Animal Production Program, Division of Cancer Treatment, National Cancer Institute, Frederick, Md. Mice were maintained in microisolators under specific pathogen-free conditions.

Antigens. Purified FHA and pertactin were purchased from Smith Kline Biologicals, Rixensart, Belgium. Pertussis toxoid, a genetically inactivated toxoid, was purchased from Biocine Sclavo, Siena, Italy.

Preparation of microspheres. Prior to encapsulation, antigens were extensively dialyzed in 1 M NH_4HCO_3 (Sigma Chemical Co., St. Louis, Mo.) and then were lyophilized. Microspheres containing DL-PLG and either *B. pertussis* FHA, pertussis toxoid, or pertactin were prepared under Department of Health and Human Services-Public Health Service contract number 223-91-1201 by Southern Research Institute, Birmingham, Ala. The encapsulated microspheres were characterized for size distribution, protein content, and in vitro antigen release kinetics and had properties similar to those previously described (19).

Parenteral immunizations. Mice were immunized subcutaneously along the back with either 0.5 ml of microencapsulated antigen or the same amount of unencapsulated protein adsorbed to a 1:100 dilution of Alhydrogel aluminum hydroxide gel (Superfos a/s, Vedbaek, Denmark). At different times after immunization, mice were bled from the periorbital sinus. The serum was collected by centrifugation and frozen at −70°C until analysis. Serum antibodies to mi-

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croencapsulated pertussis antigens from subcutaneously immunized mice were analyzed by enzyme-linked immunosorbent assay (ELISA) on individual mice at a single serum dilution (of 1:100) at 2 weeks postimmunization in order to ascertain that all animals were responding to immunization. However, in order to run all serum in the same assay to minimize intra-assay variation, pooled sera were analyzed at all time points to generate the data shown in Fig. 1 to 3.

Respiratory and oral immunizations. Intranasal immunization of mice that had been anesthetized with Metofane inhalant anesthesia (Pittman Moore, Chicago, Ill.) was accomplished by depositing antigen in sterile saline (25 to 50 μl) on the nares and holding the mice upright until the antigen had been inhaled as previously described (26). As a control for cross-contamination of the gastrointestinal tract during intranasal inoculation, mice were administered Evans Blue dye (Sigma) under the conditions described above. No dye was observed in the esophagus, stomach, or duodenum of these animals. Microspheres were orally administered to mice with a ball-tipped feeding needle following oral administration of 0.5 ml of 1.5% sodium bicarbonate. Antigens administered by either the respiratory or the oral route were not adsorbed to aluminum hydroxide gel.

Aerosol challenge. A 21-h culture of *B. pertussis* 18323 grown on Bordet Gengou agar was suspended in sterile phosphate-buffered saline (PBS) at a concentration of approximately 10^9 CFU/ml of inoculum. The challenge inoculum was administered to mice as an aerosol for 30 min as previously described (26). Mice were removed from the chamber 1 h after termination of the aerosol challenge, at which point viable *B. pertussis* cannot be cultured from the surface of the animals or the air of the chamber. Two mice were sacrificed upon removal from the chamber in order to determine the number of viable *B. pertussis* cells in the lungs. Lungs and tracheas from groups of four to seven adult mice were aseptically removed and homogenized in 5 ml (lungs) or 1 ml (tracheas) of sterile PBS, and 10-fold dilutions of homogenates were plated on Bordet Gengou agar in order to determine the number of recoverable bacteria. All animals tested had approximately 10^5 CFU in their lungs 1 h after aerosol challenge. Plates incubated with undiluted homogenate that had no *B. pertussis* growth were scored as having one-half of a CFU. Student's *t* test was used to test bacterial recovery data for statistical significance. Each experiment was repeated at least twice, with equivalent results. Data from representative experiments are reported.

Analysis of respiratory, gastrointestinal, and serum Ig. Bronchoalveolar lavage fluids (BAL) and nasal washes were collected by lavage of the lungs and tracheas with sterile PBS as previously described (26). Gut secretions were collected by the method of Elson et al. (8). Serum from mucosally immunized mice was collected by bleeding anesthetized animals from the brachial artery. All samples were maintained at −70°C until analyzed.

Serum and secretions from mucosally immunized mice were individually analyzed for specific antibody by an ELISA (26). Microtiter plates (Immunolon I; Dynatech Laboratories, Chantilly, Va.) coated with 5 μg of FHA, pertussis toxoid, or pertactin per ml overnight were incubated with dilutions of either mouse serum, BAL, nasal washes, or gut washes for 3 h. After washing, plates were incubated for 2 h with alkaline phosphatase-conjugated goat anti-mouse Ig or with alkaline phosphatase-conjugated goat anti-mouse IgM, IgG, or IgA (Southern Biotechnology Associates, Inc., Birmingham, Ala.). The isotype specificities and sensitivities of the alkaline phosphatase conjugates were established with a panel of purified mouse myeloma proteins (Organon Teknika, Durham, N.C.). The plates were read 30 min after addition of Sigma 104 phosphatase substrate (Sigma) with a Bio-Tek EL 312 reader (Bio-Tek Instruments, Winooski, Vt.). Specific antibody titers are expressed as the reciprocal of the endpoint dilution, calculated by extrapolation to zero from the linear part of the titration curve.

RESULTS

Microencapsulated FHA, pertussis toxoid, and pertactin maintain their immunogenicity. Encapsulation of antigen into DL-PLG microspheres involves exposure of the antigens to organic solvents, with the potential for destruction of the immunogenicity of the antigens. Therefore, the immunogenicity of the microencapsulated *B. pertussis* antigens was evaluated by analysis of specific antibody titers following parenteral immunization. Mice immunized subcutaneously with either 50 μg of FHA microencapsulated into DL-PLG microspheres or the same dose of FHA adsorbed to alum were bled at different times after injection, and their sera were titered for specific anti-FHA antibodies. High titers of IgG anti-FHA antibodies were detected in the sera of mice administered microencapsulated FHA as well as in the sera of mice administered unencapsulated FHA (Fig. 1). Comparisons of the immunogenicity of parenterally administered microencapsulated pertussis toxoid with that of unencapsulated pertussis toxoid adsorbed to alum (Fig. 2) and of the immunogenicity of microencapsulated pertactin with that of unencapsulated pertactin adsorbed to alum (Fig. 3) were done by the same procedure. While FHA, pertussis toxoid, and some pertactin microspheres were prepared with methylene chloride as a solvent, some batches of pertactin were encapsulated with ethyl acetate as a solvent, because of concerns that methylene chloride might destabilize immunogenic epitopes on this outer membrane protein. Maintenance of immunogenicity was seen for encapsulated pertussis toxoid, as well as for encapsulated pertactin made with either methylene chloride or ethyl acetate as a solvent (Fig. 3).

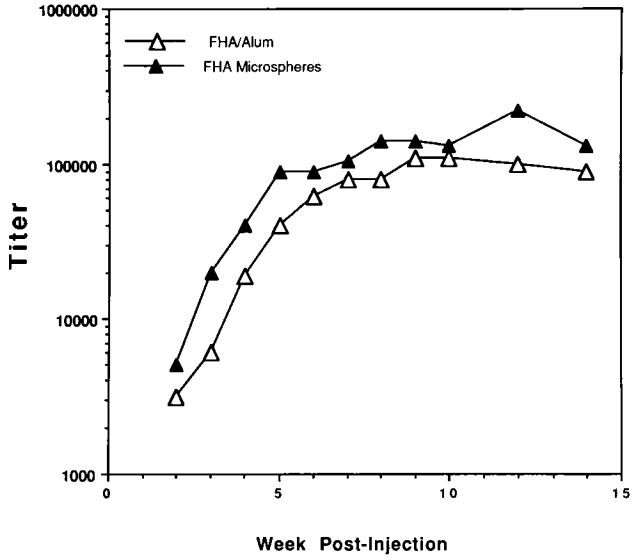

FIG. 1. IgG anti-FHA endpoint titers in the sera of mice that had received either 50 μg of FHA encapsulated in DL-PLG microspheres (▲) or 50 μg of FHA adsorbed to aluminum hydroxide gel (△) subcutaneously. Sera from five or six mice per group were individually assayed, and the mean of the endpoint titers for each group is reported.

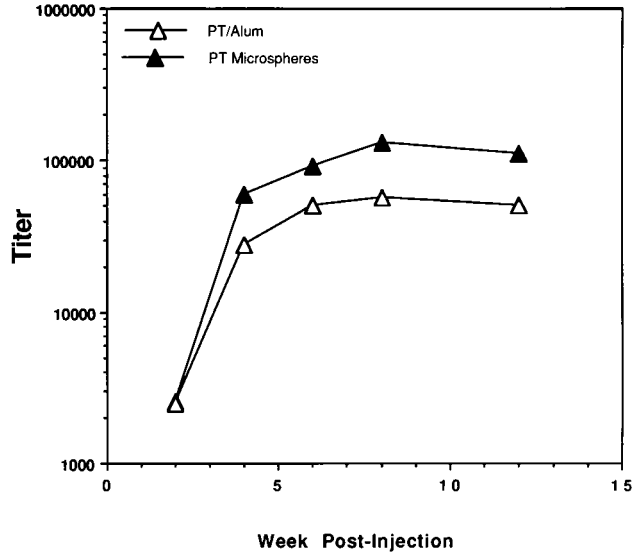

FIG. 2. IgG anti-pertussis toxin endpoint titers in the sera of mice that had received either 50 μg of pertussis toxoid encapsulated in DL-PLG microspheres (▲) or 50 μg of pertussis toxoid adsorbed to aluminum hydroxide gel (△) subcutaneously. Sera from 5 or 6 mice per group were individually assayed, and the mean of the endpoint titers for each group is reported.

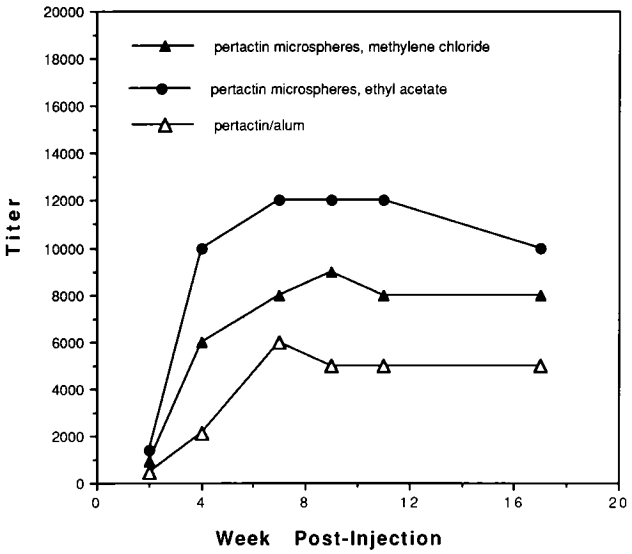

FIG. 3. IgG antipertactin endpoint titers in the sera of mice that had received either 50 μg of pertactin encapsulated in DL-PLG microspheres made with methylene chloride (▲), 50 μg of pertactin encapsulated in DL-PLG microspheres made with ethyl acetate (●), or 50 μg of pertactin adsorbed to aluminum hydroxide gel (△) subcutaneously. Sera from 5 or 6 mice per group were individually assayed, and the mean of the endpoint titers for each group is reported.

Intranasal administration of DL-PLG microspheres containing pertussis antigens elicits specific systemic and mucosal antibodies. Adult BALB/c mice were immunized with two intranasal doses of 1 or 10 μg of FHA in microspheres, given 1 month apart, and their sera and secretions were analyzed for specific IgG and IgA antibody to FHA 1 month after the last immunization. A second group of mice was immunized according to the same schedule with unencapsulated FHA. Intranasal instillation of 1-μg doses of FHA encapsulated in DL-PLG microspheres elicited high levels of IgG anti-FHA antibodies in the serum and BAL and high levels of IgA anti-FHA antibodies in the BAL of the immunized mice (Table 1). In contrast, no detectable specific antibody was found in the sera or secretions of mice administered 1 μg of unencapsulated FHA (Table 1). Higher titers of IgG and IgA anti-FHA antibodies were observed in the sera and BAL of all mice receiving 10-μg doses of microencapsulated FHA intranasally, while only one of five mice immunized intranasally with 10-μg doses of unencapsulated FHA made detectable serum and secretory antibody (Table 1). Of interest was the observation that a low but detectable IgA anti-FHA antibody response was found in the gut wash of four of five mice that had received 10-μg intranasal doses of microencapsulated FHA.

TABLE 2. Anti-pertussis toxoid antibody in sera and bronchoalveolar lavage of mice immunized intranasally with pertussis toxoid microspheres

Immunization group[a]	Anti-PT titer (no. of responders/total no.) in indicated fluid[b]			
	IgG		IgA	
	Serum	BAL	Serum	BAL
Control	<50	<2	<50	<2
10 μg of PT in PBS	52,000 (5/5)	82 (5/5)	<50	36 (3/5)
10 μg of PT in microspheres	55,000 (5/5)	218 (5/5)	<50	40 (4/5)
1 μg of PT in PBS	<50 (5/5)	<2 (5/5)	<50	<2
1 μg of PT in microspheres	25,000 (5/5)	77 (5/5)	<50	30 (5/5)

[a] Mice received two intranasal immunizations, 4 weeks apart, with either antigen in PBS or antigen encapsulated in microspheres.
[b] Sera and secretions were collected 4 weeks after the last immunization. Specific antibody was not detected in the nasal wash or gut wash of these animals. These data are representative of results from at least two independent experiments. PT, pertussis toxoid.

Intranasal instillation of 1-μg doses of pertussis toxoid encapsulated in DL-PLG microspheres elicited high levels of antigen-specific serum IgG antibodies and respiratory IgA antibodies, in contrast to the lack of specific antibody detected following intranasal administration of 1-μg doses of unencapsulated pertussis toxoid (Table 2). Administration of either 10 μg of pertussis toxoid in DL-PLG microspheres or 10 μg of unencapsulated pertussis toxoid resulted in the production of serum IgG antibodies and respiratory IgA antibodies to pertussis toxoid (Table 2). High levels of antigen-specific serum IgG antibodies and respiratory IgA antibodies were also seen in mice intranasally administered 10 μg of pertactin encapsulated in DL-PLG microspheres, but not in animals receiving the same dose of unencapsulated pertactin (Table 3).

Intranasal administration of DL-PLG microspheres containing pertussis antigens decreases experimental pertussis infection. Adult BALB/c mice were immunized with two intranasal doses of 1 or 10 μg of FHA in microcapsules, given 1 month apart and challenged 3 weeks after the last immunization, and had a 2- to 3-$\log_{10}$ CFU reduction ($P < 0.01$ for both 1- and 10-μg dose microsphere groups compared with controls) in bacterial recovery from their lungs, as well as a 1- to 2-$\log_{10}$ CFU reduction ($P < 0.01$ for both 1- and 10-μg dose

TABLE 1. Anti-FHA antibody in sera and secretions of mice immunized intranasally with FHA microspheres

Immunization group[a]	Anti-FHA titer (no. of responders/total no.) in indicated fluid[b]							
	IgG				IgA			
	Serum	BAL	Nasal wash	Gut wash	Serum	BAL	Nasal wash	Gut wash
Control	<100	<2	<2	<2	<100	<2	<2	<2
10 μg of FHA in PBS	70,000 (1/5)	225 (1/5)	12 (1/5)	<2	<2	25 (1/5)	<2	<2
10 μg of FHA in microspheres	330,000 (5/5)	1,340 (5/5)	14 (2/5)	<2	1,960 (5/5)	300 (5/5)	14 (2/5)	15 (4/5)
1 μg of FHA in PBS	<100	<2	<2	<2	<2	<2	<2	<2
1 μg of FHA in microspheres	90,000 (5/5)	470 (5/5)	26 (1/5)	<2	625 (2/5)	100 (5/5)	9 (1/5)	<2

[a] Mice received two intranasal immunizations, 4 weeks apart, with either FHA in PBS or FHA encapsulated in microspheres.
[b] Sera and secretions were collected 4 weeks after the last immunization. The mean endpoint titers of responding mice are reported. These data are representative of results from at least two independent experiments.

TABLE 3. Antipertactin antibody in sera and secretions of mice immunized intranasally with pertactin microspheres

Immunization group[a]	Antipertactin titer (no. of responders/total no.) in indicated fluid[b]			
	IgG		IgA	
	Serum	BAL	Serum	BAL
Control	<50	<2	<50	<2
10 μg of pertactin in PBS	<50	<2	<50	<2
10 μg of pertactin in microspheres	21,000 (6/6)	160 (6/6)	<50	50 (6/6)

[a] Mice received two intranasal immunizations, 4 weeks apart, with either antigen in PBS or antigen encapsulated in microspheres.
[b] Sera and secretions were collected 4 weeks after the last immunization. Specific antibody was not detected in the nasal wash or gut wash of these animals. These data are representative of results from at least two independent experiments.

TABLE 5. Bacterial recovery following *B. pertussis* aerosol challenge of mice immunized intranasally with pertussis toxoid microspheres

Immunization group[a]	Log_{10} CFU (no. infected/total no.) from[b]:	
	Lungs	Tracheas
1 μg of PT in microspheres[c]	4.34 ± 1.48 (4/4)	2.69 ± 1.25 (4/4)
1 μg of PT in saline	6.44 ± 0.10 (5/5)	4.73 ± 0.36 (5/5)
10 μg of PT in microspheres	4.67 ± 0.83 (5/5)	3.69 ± 0.77 (5/5)
10 μg of PT in saline	4.62 ± 0.69 (4/4)	2.29 ± 0.82 (5/5)
Unimmunized infected controls	6.31 ± 0.17 (5/5)	4.49 ± 0.56 (4/4)

[a] Mice received two intranasal immunizations, 4 weeks apart, with either antigen in PBS or antigen encapsulated in microspheres and were challenged 4 weeks following the last immunization with an aerosol of virulent *B. pertussis*.
[b] CFU values are means ± standard deviations.
[c] PT, pertussis toxoid.

microsphere groups compared with controls) in bacterial recovery from their tracheas, in comparison with the bacterial recoveries from the lungs of unimmunized, infected control mice (Table 4). Mice administered 1- or 10-μg intranasal doses of FHA in saline prior to infection had bacterial recoveries that were not statistically different from those of unimmunized infected control mice (Table 4).

Administration of three oral 100-μg doses of FHA encapsulated in DL-PLG microspheres (given 1 month apart and analyzed either 10 and 20 days or 30 and 60 days after the last oral dose) neither elicited consistently detectable anti-FHA antibodies in serum and secretions, including gut and respiratory secretions, nor decreased bacterial recoveries from lungs and tracheas following experimental respiratory infection (data not shown).

Intranasal administration of 1-μg doses of pertussis toxoid encapsulated in DL-PLG microspheres prior to infection resulted in decreased bacterial recoveries in comparison with bacterial recoveries from mice administered 1-μg doses of unencapsulated pertussis toxoid (Table 5). Administration of either 10 μg of pertussis toxoid in DL-PLG microspheres or 10 μg of unencapsulated pertussis toxoid prior to infection also reduced bacterial recoveries from the lungs and tracheas in comparison with bacterial recoveries from unimmunized infected mice (Table 5). A 2-log_{10} CFU reduction in bacterial recoveries from the lungs and tracheas following aerosol infection was also seen in mice intranasally administered 10 μg of pertactin encapsulated in DL-PLG microspheres, but not in animals receiving the same dose of unencapsulated pertactin (Table 6). Reductions in bacterial recoveries from the lungs and tracheas were observed in mice receiving pertactin microcapsules made with methylene chloride as a solvent, as well as those made with ethyl acetate as a solvent (Table 6).

Coadministration of microencapsulated FHA, pertussis toxoid, and pertactin. Microencapsulated pertussis antigens were coadministered intranasally in order to ascertain the effects of immunization with combinations of antigens on bacterial recoveries following aerosol challenge. Two intranasal doses of a combination of 1 μg of microencapsulated FHA, 1 μg of microencapsulated pertussis toxoid, and 1 μg of microencapsulated pertactin were administered 1 month apart, and these mice were challenged 3 weeks after the last immunization. A statistically significant difference in bacterial recoveries from the lungs and tracheas was observed in mice receiving the coadministered FHA, pertussis toxoid, and pertactin microspheres in comparison with the bacterial recoveries from mice receiving microspheres containing the same dose of only FHA, pertussis toxoid, or pertactin ($P < 0.05$ [Table 7]).

TABLE 4. Bacterial recovery following *B. pertussis* aerosol challenge of mice immunized intranasally with FHA microspheres

Immunization group[a]	Log_{10} CFU (no. infected/total no.) from[b]:	
	Lungs	Tracheas
1 μg of FHA in microspheres	3.63 ± 1.03 (5/5)	1.68 ± 1.11 (3/5)
1 μg of FHA in saline	6.13 ± 0.20 (5/5)	4.32 ± 0.50 (5/5)
10 μg of FHA in microspheres	2.34 ± 0.96 (3/5)	1.40 ± 0.66 (3/5)
10 μg of FHA in saline	6.14 ± 0.20 (5/5)	4.72 ± 0.08 (5/5)
Unimmunized infected controls	6.30 ± 0.17 (5/5)	4.60 ± 0.39 (5/5)

[a] Mice received two intranasal immunizations, 4 weeks apart, with either antigen in PBS or antigen encapsulated in microspheres and were challenged 4 weeks following the last immunization with an aerosol of virulent *B. pertussis*.
[b] CFU values are means ± standard deviations.

TABLE 6. Bacterial recovery following *B. pertussis* aerosol challenge of mice immunized intranasally with pertactin microspheres

Immunization group[a]	Log_{10} CFU (no. infected/total no.) from[b]:	
	Lungs	Tracheas
10 μg of pertactin in microspheres[c]	4.61 ± 0.20 (6/6)	2.16 ± 1.07 (6/7)
10 μg of pertactin in saline	6.67 ± 0.09 (7/7)	4.76 ± 0.19 (7/7)
Unimmunized infected controls	6.77 ± 0.12 (7/7)	4.84 ± 0.35 (7/7)
10 μg of pertactin in microspheres[d]	3.12 ± 1.25 (3/4)	1.64 ± 0.87 (3/5)
1 μg of pertactin in microspheres[d]	3.30 ± 1.17 (4/5)	2.05 ± 0.70 (5/5)
10 μg of pertactin in saline	5.85 ± 0.18 (5/5)	3.59 ± 0.57 (5/5)
1 μg of pertactin in saline	6.04 ± 0.13 (5/5)	4.37 ± 0.22 (5/5)
Controls	5.97 ± 0.22 (5/5)	4.16 ± 0.43 (3/3)

[a] Mice received two intranasal immunizations, 4 weeks apart, with either antigen in PBS or antigen encapsulated in microspheres and were challenged 4 weeks following the last immunization with an aerosol of virulent *B. pertussis*.
[b] CFU values are means ± standard deviations.
[c] Microspheres made with methylene chloride as a solvent.
[d] Microspheres made with ethyl acetate as a solvent.

TABLE 7. Bacterial recovery following *B. pertussis* aerosol challenge of mice immunized intranasally with combinations of microspheres

Immunization group[a]	Log_{10} CFU (no. infected/total no.) from[b]:	
	Lungs	Tracheas
FHA + PT + pertactin in microspheres[c]	1.76 ± 0.67 (2/7)	0.74 ± 0.11 (1/7)
FHA + PT in microspheres	4.12 ± 0.66 (5/5)	2.43 ± 1.09 (4/5)
FHA + pertactin in microspheres	2.10 ± 0.81 (2/5)	0.97 ± 0.54 (1/5)
PT + pertactin in microspheres	3.59 ± 1.52 (4/5)	1.57 ± 0.84 (3/5)
FHA in microspheres	4.22 ± 1.51 (4/5)	3.37 ± 0.75 (5/5)
PT in microspheres	2.98 ± 1.33 (4/5)	2.31 ± 0.91 (4/5)
Pertactin in microspheres	3.18 ± 0.36 (5/5)	1.88 ± 1.08 (4/5)
Unimmunized infected controls	6.03 ± 0.17 (7/7)	4.21 ± 0.23 (7/7)

[a] Mice received two intranasal immunizations, 4 weeks apart, with either antigen in PBS or antigen encapsulated in microspheres and were challenged 4 weeks following the last immunization with an aerosol of virulent *B. pertussis*.
[b] CFU values are means ± standard deviations.
[c] PT, pertussis toxoid.

DISCUSSION

The maintenance of immunogenicity following encapsulation is critical to the successful use of microencapsulated proteins as mucosal vaccines. Exposure of proteins to be encapsulated in DL-PLG to organic solvents can potentially denature critical immunoprotective epitopes. In vivo hydrolysis of the DL-PLG polymer may result in a highly acidic microenvironment inside the microsphere, which can also destroy the immunogenicity of the encapsulated antigen (9). We have demonstrated that three different pertussis antigens retain their immunogenicity following microencapsulation in DL-PLG microspheres, as determined by the serum antibody responses detected after subcutaneous administration of antigen. These three bacterial antigens differ greatly in size and physical properties. FHA, a major adhesin of *B. pertussis* (12), is a 200-kDa secreted protein with a hydrophobic face that self associates into a 50-nm-long filamentous structure (17) and has a distinct carbohydrate-binding site, an integrin-binding site, and a heparin-binding site (20). Pertactin is a 69-kDa proline-rich outer membrane protein that is released from the cell membrane following heating; pertactin also contains an integrin-binding site and has been shown to mediate binding to eukaryotic cell lines in vitro. Pertussis toxin is a hexameric protein, composed of an enzymatically active S1 subunit and a pentameric B oligomer composed of four different subunits (28). Pertussis toxin binds to eukaryotic cell membranes via the B oligomer, prior to S1-mediated ADP ribosylation of G proteins (11, 30, 31). Both pertussis toxin and pertactin have been shown to contain critical conformational epitopes that are bound by protective monoclonal antibodies.

We have previously shown that mucosal immunization with 50- to 100-μg doses of unencapsulated *B. pertussis* FHA elicits specific local immune responses and decreases bacterial recoveries from the lungs and tracheas in a mouse model of respiratory *B. pertussis* infection (26). In the experiments presented in this paper, microencapsulation of FHA showed a clear adjuvant effect when presented via the intranasal route, eliciting protective immune responses at amounts of antigen 10- to 100-fold lower than those required to elicit protection with unencapsulated FHA (24). Intranasal doses of 1 and 10 μg of microencapsulated FHA decreased *B. pertussis* respiratory infection and stimulated antigen-specific IgG and IgA antibodies in serum and secretions, whereas the same doses of unencapsulated antigen failed to protect against infection (Table 4) and elicited no detectable specific antibody in the majority of immunized mice (Table 1). Such enhanced protective immunity was also observed for microencapsulated pertussis toxoid and microencapsulated pertactin (Tables 2, 3, 5, and 6). Thus, respiratory immunization with a microencapsulated antigen can protect against bacterial colonization of the lungs and trachea. A correlation between the ability of intranasal immunization with microgram doses of microencapsulated pertussis antigens to elicit high-titer specific antibody in the serum and secretions of immunized mice and the ability to decrease experimental *B. pertussis* infection in the respiratory tract was observed. At this time, the exact mechanisms that contribute to this enhanced respiratory immunogenicity of antigens encapsulated in DL-PLG microspheres are not well understood but may include stimulation of macrophage upon uptake of antigen-containing microspheres (6), prolonged slow release of antigen to the immune system upon hydrolysis of the DL-PLG polymer (Fig. 1 to 3), and targeting of macrophage-containing microspheres to both peripheral lymph nodes as well as to mucosal follicles.

In contrast, oral administration of 100-μg doses of microencapsulated FHA from the same batches failed to consistently elicit detectable specific antibody in the serum, lung, or gut secretions of immunized mice. Successful induction of mucosal immune responses following oral administration of DL-PLG microspheres has been reported for several microencapsulated antigens, including staphylococcal enterotoxin B (5), enterotoxigenic *Escherichia coli* fimbrial adhesins (4, 24), and ovalbumin (2). Direct injection of 100-μg quantities of FHA into the lumen of the duodenum has also been shown to elicit a protective immune response to respiratory *B. pertussis* challenge (26). Failure of microencapsulated FHA to stimulate a protective mucosal response via the oral route, while maintaining immunogenicity via the respiratory route, may reflect, among other things, insufficient uptake of microencapsulated antigen across the Peyer's patch. Less than 1% of an oral dose of DL-PLG microspheres successfully reaches the Peyer's patch (5).

Respiratory immunization with antigens, especially in particulate form, has been a successful strategy for the induction of both systemic and mucosal immune responses in several systems, including microspheres (6, 19), proteosomes (22), liposomes (1), and antigens sorbed to polystyrene beads. Antigens delivered to the lungs can be taken up by lung macrophages, including airway, interstitial, and alveolar macrophages, as well as by dendritic cells (10, 14). It has been demonstrated that lung macrophages that have phagocytosed particulate antigens can translocate from the lung to regional tracheobronchial lymph nodes (10); antigen-bearing macrophages that reach the lung have also been demonstrated to traffic to the mucosal follicles of the bronchus-associated lymphoid tissue (23). Thus, the site to which antigen-presenting cells that have taken up microspheres traffic likely influences the quality of the elicited B- and T-lymphocyte immune responses, especially in regard to the antibody isotype(s) and lymphocyte effector function(s) that are observed.

Of interest was the observation that the intranasal administration of a combination of 1 μg of each of the microencapsulated pertussis antigens was more effective in reducing bacterial infection than administration of any single microencapsulated antigen (Table 7). This suggests a cooperative protective effect of multiple pertussis antigens administered to a mucosal site. Such cooperative protection has been previously reported for parenterally injected pertactin and FHA in pro-

tection against an intracerebral challenge of virulent *B. pertussis* (21). In a randomized, placebo-controlled clinical trial of two parenteral acellular pertussis vaccines, the addition of FHA to pertussis toxoid appears to have provided benefit in decreasing infection, in addition to the prevention of severe clinical disease attributed to pertussis toxoid alone (29). FHA is a high-molecular-weight secreted adhesin. A mutant defective in FHA was shown by Kimura and colleagues to have a significantly reduced, but not completely abolished, ability to colonize the tracheas of mice early in infection (12). Studies by other investigators in in vitro adhesion systems suggest additional roles for pertactin and pertussis toxin in mediating adhesion of *B. pertussis* to eukaryotic cells (15, 32). These studies taken together with the data in Table 7 suggest that *B. pertussis* elaborates multiple adhesins and that effective abrogation of colonization requires immunity to each of these adhesins.

The exact mechanisms of protective immunity to *B. pertussis* respiratory infection by respiratory administration of microencapsulated antigen remain to be elucidated and may also involve antigen-specific T cells, phagocytes, and cytokines in addition to specific antibody. Intranasal immunization with biodegradable microspheres is a useful system for determining the optimal conditions for eliciting a potent protective mucosal immune response against other respiratory pathogens in addition to *B. pertussis*, as well as a useful tool to further our understanding of protective immunity in the respiratory tract.

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