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Mucosal Immunization with Filamentous Hemagglutinin Protects against *Bordetella pertussis* Respiratory Infection

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Mucosal immunization of mice with purified *Bordetella pertussis* filamentous hemagglutinin (FHA), by either the respiratory or the gut route, was found to protect against *B. pertussis* infection of the trachea and lungs. Intranasal immunization of BALB/c and (C57BL/6 × C3H/HeN)F_1 adult female mice with FHA prior to *B. pertussis* aerosol challenge resulted in a 2 to 3 log reduction in number of bacteria recovered from the lungs and the tracheas of immunized mice in comparison to unimmunized controls. Intraduodenal immunization of adult mice with FHA before infection also resulted in approximately a 2 log reduction in the recovery of bacteria from the lungs and the tracheas of immunized mice in comparison to unimmunized controls. Immunoglobulin A and immunoglobulin G anti-FHA were both detected in bronchoalveolar lavage fluids of mucosally immunized mice. Limiting dilution analysis revealed a 60-fold increase in the frequency of FHA-specific B cells isolated from the lungs of mice immunized intranasally with FHA in comparison to unimmunized control mice. These data suggest that both gut and respiratory mucosal immunization with a major adhesin of *B. pertussis* generates a specific immune response in the respiratory tract that may serve as one means of mitigating subsequent *B. pertussis* respiratory infection.

Pertussis is a highly contagious human respiratory disease, typified by episodes of paroxysmal coughing, that is caused by the gram-negative bacillus *Bordetella pertussis*. *B. pertussis* infects via inhalation of aerosol droplets and preferentially associates with the cilia of the respiratory epithelium lining the nasopharynx, trachea, and bronchial tree. During the course of disease, *B. pertussis* infection remains localized to the respiratory tract and does not progress to bacteremia or meningitis (4, 6).

The localization of *B. pertussis* infection to the ciliated epithelium of the respiratory tract suggests that adherence of this bacterium to cilia is a critical step in *B. pertussis* pathogenesis. Filamentous hemagglutinin (FHA) is a 220-kDa filamentous protein that is proposed to be one of the major adhesins mediating the interaction of *B. pertussis* and human cilia (17, 31).

While parenteral vaccination with either whole-cell (2) or a two-component acellular pertussis vaccine (1) has been effective in protection against clinical disease, there is, to date, no correlation between serum antibody titers to known purified pertussis antigens and clinical protection (1, 2). Therefore, aspects of immunity other than serum antibody may better reflect pertussis prophylaxis.

After natural *B. pertussis* infection, high titers of immunoglobulin A (IgA) antibodies to FHA have been detected in the nasal secretions of convalescent patients (20, 40). Naturally occurring *B. pertussis* infection has been shown to confer long-lasting protection against reinfection, while parenteral vaccination induces protection that wanes in young adulthood (18). Long-lived resistance to pertussis, therefore, may reflect the induction of persistent mucosal immunity which can be recalled at the respiratory mucosa upon subsequent infection.

After experimental respiratory infection of mice with *B. pertussis*, the bacterium has been demonstrated to be associated with the ciliated epithelium of the murine trachea (35) and bronchial tree (33). Therefore, respiratory infection of mice may be used to analyze parameters of immunity that interfere with the persistence of *B. pertussis* at the respiratory epithelium. The purpose of this study was to determine the ability of mucosal immunization with FHA to stimulate an antigen-specific immune response in the respiratory tract that could protect against *B. pertussis* infection.

MATERIALS AND METHODS

Mice. BALB/cAnNcR or (C57/B16 × C3H/HeN)F_1 adult mice (B6C3 mice), 5 to 8 weeks of age, and BALB/cAnNcR newborn 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. FHA, purified from *B. pertussis* Tohama I, was kindly provided by Alan Kimura and James Cowell, Praxis Biologics, Rochester, N.Y., and by Jean Petre and Carine Capiau, Smith Kline Biologicals, Rixensart, Belgium. The preparations of FHA used in these studies ran predominantly as single bands of 220 kDa on sodium dodecyl sulfate (SDS)-polyacrylamide gels, had less than 0.005% pertussis toxin contamination, and contained approximately 0.05% endotoxin (17) or were purified from a strain from which the pertussis toxin gene had been deleted and which contained approximately 0.0006% endotoxin (Smith Kline preparation) as determined by the *Limulus* amoebocyte lysate assay. Both preparations yielded similar results in these studies.

Intranasal immunization. Antigen in sterile saline (50 μl) was deposited on the nares of mice that had been anesthetized with Metofane inhalant anesthesia (Pitman Moore, Chicago, Ill.), and the mice were held upright until the antigen had been inhaled. As a control for cross-contamination of the gastrointestinal tract during intranasal inoculation, mice were administered Evans blue dye (Sigma Chemical Co., St. Louis, Mo.) under the conditions described

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above. Examination of the respiratory tracts and gastrointestinal tracts of these animals showed that the dye remained localized to the trachea, bifurcation of the bronchi, and upper portions of the lungs; no dye was observed in the esophagus, stomach, or duodenum.

Intraduodenal immunization. Mice were anesthetized intraperitoneally with 0.35 ml of a 2% solution of 2,2,2-tribromoethanol (Aldrich Chemical Co., Milwaukee, Wis.). A midline incision below the sternum was made, and 0.2 ml of antigen in sterile saline was injected into the lumen of the duodenum by using a 30-gauge needle (Becton Dickinson and Co., Rutherford, N.J.) as previously described (15). The injection site was inspected to ensure that there was no leakage, and the incisions were sutured.

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 (37). 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 chamber. Two mice were sacrificed upon their removal from the chamber to determine the number of viable *B. pertussis* cells in the lungs. All animals tested had approximately 10^5 CFU in their lungs at 1 h after aerosol challenge. Lungs and tracheas from adult mice were aseptically removed and homogenized in sterile PBS, and dilutions of homogenates were plated on Bordet-Gengou agar to determine the number of recoverable bacteria. Plates incubated with neat homogenate that had no *B. pertussis* growth were scored as having 0.5 CFU. Student's *t* test was used to test bacterial recovery data for statistical significance.

Analysis of respiratory and serum immunoglobulin. Mice anesthetized with tribromoethanol were bled from the brachial artery, and their tracheas were cannulated with a piece of PE-50 polyethylene tubing (Clay Adams, Parsippany, N.J.) held in place by a tied loop of suture. Sterile PBS (0.5 ml) was gently instilled into the lungs and withdrawn three times. The bronchoalveolar lavage (BAL) fluid was centrifuged, and the supernatant was removed and frozen at −20°C prior to analysis. Nasal washes were collected by reversing the cannula in the trachea, slowly instilling 0.5 ml of PBS, and collecting it from the nares. BAL fluid and nasal washes were monitored for blood contamination due to processing contamination during harvest with Bililabstix (Miles Laboratories Inc.) as previously described (32). Slight blood contamination was seen in some samples, but the level of contaminating serum anti-FHA antibody that this represented was too small to affect the results.

Serum and BAL fluid were analyzed for specific antibody by an enzyme-linked immunosorbent assay (ELISA) (21). Microtiter plates (Immunolon I; Dynatech Laboratories, Chantilly, Va.), coated with 5 μg of FHA per ml overnight, were incubated with dilutions of mouse serum or BAL fluid 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 specificity and sensitivity of the alkaline phosphatase conjugates were established by using a panel of purified mouse myeloma proteins (Organon Teknika, Durham, N.C.). The plates were read 30 min after the addition of Sigma 104 phosphatase substrate (Sigma), by using a Bio-Tek EL 312 reader (Bio-Tek Instruments, Winooski, Vt.).

Immunoblot analysis was used to demonstrate the specificity of antibodies elicited by immunization with the preparations of FHA described above. Tricine urea extracts of *B. pertussis* 18323 (12) were analyzed electrophoretically on an SDS-polyacrylamide gel with a 4 to 20% polyacrylamide gradient (Integrated Separation Systems, Hyde Park, Mass.) and transferred to nitrocellulose. Strips of the nitrocellulose were incubated with either a mixture of the monoclonal antibodies MO8X3C and 12.1D3 as a positive control for FHA or serum or BAL fluid from mice immunized intranasally with FHA, and they were then developed with alkaline phosphatase-conjugated goat anti-mouse Ig as previously described (37). MO8X3C recognizes the same proteolytic fragments of FHA as does MO8X3E (37) and was a gift from J. Kenimer, U.S. Food and Drug Administration. 12.1D3 recognizes proteolytic fragments of FHA not seen by MO8X3C (8) and was generously provided by C. Locht, Institut Pasteur, Lille, France.

Total IgG and IgA titers were determined by incubating plates coated with 5 μg of unlabelled goat anti-mouse Ig (Southern Biotechnology Associates) per ml with dilutions of BAL fluid or nasal wash for 3 h. After being washed the plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG or IgA and developed as described above. Anti-FHA titers as well as total Ig titers are expressed as the reciprocal of the endpoint dilution, calculated by extrapolation to zero from the linear part of the titration curve.

Limiting dilution analysis. Lymphocytes were isolated from the lungs of B6C3 mice by mild enzymatic treatment (34) or from the spleens of mice by mechanical dispersion and were analyzed in limiting dilution microcultures (in the absence of dendritic cells) as previously described (36). Supernatants were collected after 7 days of culture and analyzed by ELISA for the production of anti-FHA antibodies. The percentage of B lymphocytes in each preparation was determined by staining with fluorescein-conjugated goat anti-mouse total Ig (Southern Biotechnology Associates). Frequencies of FHA-specific B cells were calculated by Poisson analysis.

RESULTS

Time course of bacterial recovery. The initial bacterial recovery from the lungs of unimmunized, aerosol-infected adult mice, determined 1 h after aerosol challenge, was 10^5 CFU of *B. pertussis* (Fig. 1). An increase in bacterial recovery, to 10^7 CFU, was observed 1 week postinfection and declined thereafter (Fig. 1). Therefore, protection, measured by comparing the bacterial recovery from the lungs of immunized mice with that of unimmunized mice, was determined in the reported experiments at 1 week postinfection.

Parenteral immunization of neonates with FHA prevents leukocytosis and death due to *B. pertussis* infection. Neonatal BALB/c mice, immunized intraperitoneally with 16 μg of FHA on days 5 and 12 postpartum and aerosol challenged on day 18 postpartum with *B. pertussis* 18323, had a mean count of 23,600 leukocytes per μl of blood 19 days postinfection, with 12 of 12 neonates surviving 22 days postinfection. In contrast, neonates immunized with tetanus toxoid prior to infection had a mean count of 128,000 leukocytes per μl 19 days postinfection, with 2 of 12 neonates surviving.

Intranasal immunization with FHA reduces respiratory *B. pertussis* infection and elicits specific antibody in the respira-

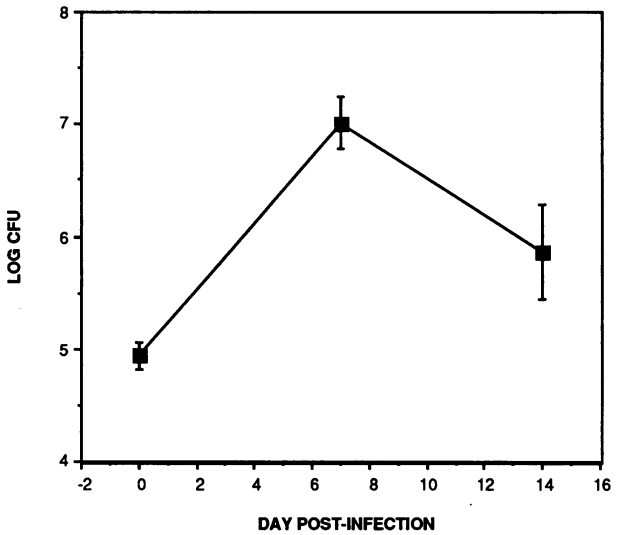

FIG. 1. Recovery of *B. pertussis* from the lungs of adult BALB/c mice after aerosol infection with *B. pertussis* 18323. Geometric means and standard deviations of bacterial recoveries from five to seven mice per group are shown.

tory tract. Adult BALB/c mice immunized with two intranasal doses of 100 μg of FHA, given 1 week apart, and challenged 3 weeks after the last immunization, had a 2 to 3 log reduction ($P < 0.01$) in bacterial recovery from their lungs, as well as a 1 to 2 log reduction ($P < 0.01$) in bacterial recovery from their tracheas, in comparison to the bacterial recoveries from the lungs of unimmunized, infected control mice (Table 1).

B6C3 mice, used in limiting dilution experiments, were also analyzed. Immunized B6C3 mice had a 3 log reduction ($P < 0.01$) in bacterial recovery from their lungs and a 2 log reduction ($P < 0.01$) in bacterial recovery from their tracheas in comparison to those recoveries in unimmunized controls (Table 1). In the experiments described in Table 1, 25 and 63% of the mice in immunized groups 1 and 2, respectively, completely cleared the infection from their tracheas.

Analysis of the sera and BAL fluid collected from intranasally immunized mice on the day of aerosol challenge revealed detectable titers of anti-FHA antibodies that increased 1 week after aerosol challenge (Table 2). IgG anti-FHA antibody was detected in both the serum and BAL fluid, while IgA anti-FHA was detected only in the BAL fluid. IgG anti-FHA was detected in nasal wash fluid only after immunization and aerosol challenge.

TABLE 2. Detection of anti-FHA antibody in sera and secretions of intranasally immunized mice

Weeks after infection	Titer[a]					
	Serum		BAL fluid		Nasal wash	
	IgG	IgA	IgG	IgA	IgG	IgA
Unimmunized mice						
0	<100	<100	<2	<2	<2	<2
1	<100	<100	<2	<2	<2	<2
2	<100	<100	<2	<2	<2	<2
Immunized mice[b]						
0	5,000	<100	150	20	<2	<2
1	50,000	<100	500	50	10	<2
2	20,000	<100	100	20	5	<2

[a] Reciprocal of the endpoint dilution of a pool from five B6C3 mice, calculated by extrapolation to zero from the linear portion of the titration curve. IgM anti-FHA was not detected in any of these samples.
[b] Mice were immunized intranasally with 100 μg of FHA at 3 and 4 weeks before *B. pertussis* aerosol challenge.

Immunoblot analysis was used to demonstrate the specificity of serum and BAL fluid antibodies to FHA. Tricine-urea extracts of *B. pertussis* 18323 separated on an SDS-polyacrylamide gel with a 4 to 20% polyacrylamide gradient and stained with Coomassie blue revealed multiple bands between 15 and 200 kDa, with numerous bands detected between 15 and 43 kDa (data not shown). Immunoblots of these extracts developed with the sera and BAL fluid from mice immunized intranasally with FHA recognized only the same bands visualized by the MO8X3C and 12.1D3 monoclonal antibodies to FHA (Fig. 2).

Since the concentration of specific antibody in the BAL fluid can be affected by variations in fluid recovery during lavage, we attempted to normalize the concentration of specific IgG and IgA anti-FHA antibody to the concentration of total IgG and total IgA in the BAL fluid. However, quantitation of total antibody in BAL fluids at different times after aerosol challenge revealed an increase in both total IgG as well as total IgA in unimmunized, aerosol infected mice (Table 3). However, little or no increase in total antibody levels in BAL fluids could be detected in intranasally immunized mice after aerosol challenge. Because of this observed variation in total antibody levels, specific anti-FHA titers in BAL fluids were not normalized to total antibody titers.

Limiting dilution analysis of lymphocytes isolated from the lungs and tracheas of intranasally immunized B6C3 mice

TABLE 1. Bacterial recovery after aerosol challenge with *B. pertussis* 18323

Expt	Immunization	Bacterial recovery (log CFU)[a]		No. of infected organs/total	
		Lungs	Tracheas	Lungs	Tracheas
1: BALB/c mice	None (control)	6.68 ± 0.11	4.59 ± 0.18	8/8	8/8
	Intranasal[b]	4.82 ± 0.97 (1.86[c], $P < 0.01$)	1.89 ± 1.06 (2.22[c], $P < 0.01$)	8/8	6/8
2: B6C3 F_1 mice	None (control)	6.18 ± 0.14	3.09 ± 0.74	8/8	8/8
	Intranasal[b]	3.16 ± 1.45 (3.02[c], $P < 0.01$)	0.80 ± 1.20 (2.29[c], $P < 0.01$)	6/8	3/8

[a] Geometric means and standard deviations of bacterial recoveries from eight to nine mice per group are shown.
[b] Mice were immunized intranasally with 100 μg of FHA at 3 weeks and 4 weeks before *B. pertussis* aerosol challenge.
[c] Log reduction from control.

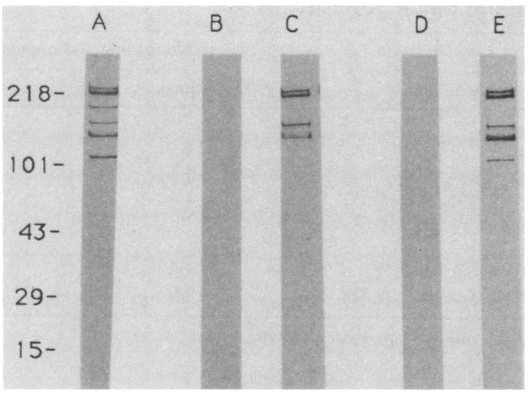

FIG. 2. Western blots of sera and BAL fluids from mice immunized intranasally with FHA; these immunoblots were performed by using the same samples analyzed in Table 1. Figure 2 is representative of the immunoblot results obtained from five immunized and three unimmunized mice. Tricine-urea extracts of *B. pertussis* 18323 were resolved electrophoretically, transferred to nitrocellulose, and developed as described in Materials and Methods. Lanes: A, reacted with both monoclonal antibodies MO8X3C and 16.1D3; B, reacted with a 1:4 dilution of BAL fluid from unimmunized mice; C, reacted with a 1:4 dilution of BAL fluid from immunized mice; D, reacted with a 1:100 dilution of serum from unimmunized mice; E, reacted with a 1:100 dilution of serum from immunized mice.

demonstrated a 60-fold increase in FHA-specific B cells when compared to lymphocytes from unimmunized controls (Table 4). However, no increase was observed in the frequency of FHA-specific splenic B lymphocytes from immunized mice when compared to unimmunized controls (Table 4).

Gut immunization with FHA protects against respiratory *B. pertussis* infection. Since intranasal immunization was successful in eliciting protective immunity in the lung, we wished to determine if immunization of the gut with FHA could disseminate a protective immune response to the respiratory tract. Mice immunized with 100 μg of FHA intraduodenally 3 weeks prior to aerosol challenge had approximately a 0.5 log reduction in bacterial recoveries from the lungs and tracheas compared to recoveries from unimmunized infected controls, although this reduction was not statistically significant (Table 5). Mice immunized with two intraduodenal doses of 100 μg of FHA, given 1 week apart, and challenged 3 weeks after the last immunization showed a 2 log reduction in bacterial recoveries from the lungs ($P < 0.01$) and tracheas ($P < 0.05$) (Table 5).

IgG anti-FHA was detected in both sera and BAL fluids after intraduodenal immunization; after aerosol challenge of immunized mice, IgA anti-FHA and IgG anti-FHA were detected in both sera and BAL fluids (Table 6).

TABLE 3. Total IgG and IgA titers in BAL fluids in immunized and unimmunized mice after aerosol infection[a]

Mice	Wk post-aerosol challenge	Titer			
		Expt 1[b]		Expt 2	
		Total IgG[c]	Total IgA	Total IgG	Total IgA
Unimmunized	0	150	50	100	30
	1	900	200	150	60
	2	1,300	700	1,500	400
Immunized[d]	0	300	450	200	200
	1	500	700	150	90
	2	500	450	250	150

[a] Total IgG and IgA titers were measured as described in Materials and Methods.
[b] Total IgG and IgA titers in experiment 1 were measured in the same BAL fluid samples used for the determination of specific anti-FHA antibody titers in Table 2.
[c] Reciprocal of the endpoint dilution of a pool from five B6C3 mice, calculated by extrapolation to zero from the linear portion of the titration curve.
[d] Immunized intranasally with 100 μg of FHA at 3 and 4 weeks before *B. pertussis* aerosol challenge.

TABLE 4. Frequency of FHA-specific B cells after intranasal immunization with FHA

Treatment group	No. of FHA-specific cells per 10^6 B lymphocytes from[a]:	
	Lungs	Spleens
Unimmunized	<1.6	3.2
Immunized[b]	63.0	0.6

[a] Lymphocytes isolated from the lungs and spleens of B6C3 mice were cultured at the limiting dilution, and their supernatants were analyzed for the production of specific antibody to FHA. Frequencies of FHA-specific B cells were calculated by using Poisson statistics.
[b] Immunized intranasally with 100 μg of FHA 3 weeks prior to cell isolation and culture.

DISCUSSION

We have demonstrated that mice immunized mucosally with purified FHA are protected against respiratory infection with *B. pertussis*. Adult mice immunized intranasally with FHA prior to aerosol challenge had a 2 to 3 $\log_{10}$ CFU decrease in bacterial recoveries from their lungs and tracheas in comparison to unimmunized controls (Table 1). IgG anti-FHA was detected in the sera, and both IgG and IgA anti-FHA were detected in the BAL fluids of intranasally immunized mice on the day of aerosol challenge; specific serum antibodies to FHA further increased in titer 1 week after immunized mice had been infected with an aerosol of *B. pertussis* (Table 2). However, antibodies to FHA were not detected in unimmunized, infected animals at either 1 or 2 weeks after infection. Only antibodies specific for FHA were elicited by intranasal immunization with the preparations of FHA used in these experiments; therefore, the decreased infection observed after mucosal immunization with FHA was indeed due to immunity elicited by FHA and not to an immunogenic contaminant of the preparations of antigen used. Parenteral immunization with this same preparation of FHA prior to aerosol challenge also prevented leukocytosis and death in neonatal mice.

Kimura et al. previously demonstrated a 1 to 2 log decrease in bacterial recovery after parenteral immunization of adult mice with two 8-μg doses of FHA (17). This decrease was accompanied by high titers of serum IgG anti-FHA in the absence of IgA anti-FHA. Since IgG has been shown to transude from the serum into the lungs (37, 39), transudation of serum antipertussis antibodies to the lungs is likely to be the mechanism of antibody-mediated protection against infection elicited by parenterally administered pertussis vaccines.

However, parenteral vaccination with either inactivated viral vaccines (e.g., inactivated polio) or killed bacterial vaccines (e.g., whole-cell pertussis) elicits little or no spe-

TABLE 5. Bacterial recovery after aerosol challenge with *B. pertussis* 18323

Group	Bacterial recovery (log CFU)[a]		No. of infected organs/total	
	Lungs	Tracheas	Lungs	Tracheas
Expt 1				
Control	6.37 ± 0.23	4.82 ± 0.34	5/5	4/4[b]
1× i.d.[c]	5.76 ± 0.75 (0.61[d], NS[e])	4.45 ± 0.59 (0.37[d], NS)	6/6	5/5[b]
2× i.d.[f]	4.63 ± 0.65 (1.74[d], $P < 0.01$)	2.63 ± 1.15 (2.19[d], $P < 0.05$)	4/4	4/4
Expt 2				
Control	6.26 ± 0.16	3.60 ± 0.38	8/8	8/8
2× i.d.[f]	4.16 ± 0.78 (2.10[d], $P < 0.01$)	1.23 ± 0.71 (2.37[d], $P < 0.01$)	9/9	4/9

[a] Geometric means ± standard deviations of bacterial recoveries from four to nine BALB/c mice per group are shown.
[b] One datum point lost because of contamination on plate.
[c] Mice immunized intraduodenally (i.d.) with a single 100-μg dose of FHA 3 weeks before *B. pertussis* aerosol challenge.
[d] Log reduction from control.
[e] NS, not significant.
[f] Mice immunized intraduodenally with 100 μg of FHA at 3 and 4 weeks before *B. pertussis* aerosol challenge.

cific mucosal antibody in the upper respiratory tract of human subjects (20, 28, 40). Thomas has shown that intramuscular immunization of adult human volunteers with killed whole pertussis vaccine caused increases in pertussis antibodies in sera but not in nasal secretions (41). In contrast, deliberate intranasal immunization of subjects with an aerosol of the same vaccine resulted in pertussis antibodies in nasal secretions but not in sera. Our data demonstrate that intranasal immunization with a single purified protein of *B. pertussis* can protect against respiratory infection. Thus, respiratory immunization with pertussis antigens elicits specific protective immunity at the site of infection.

In a randomized, placebo-controlled clinical trial of two parenteral pertussis vaccines, pertussis toxoid alone was highly effective in preventing severe clinical disease; however, the addition of FHA to pertussis toxoid appears to have provided some additional benefit in decreasing infection (38). Of note is the observation that serum antibody titers to either antigen did not correlate with clinical protection in this trial (1). Data from animal experiments suggest that the amount of specific antibody to pertussis antigens in the respiratory tract, resulting either from transudation from the serum (17, 37) or from local synthesis (Tables 2 and 4), correlates, in the case of FHA, with a decrease in *B. pertussis* infection. This hypothesis predicts, therefore, that antibodies to *B. pertussis* antigens in respiratory secretions, rather than in serum, may provide a correlate of vaccine-induced immunity to pertussis in humans.

TABLE 6. Anti-FHA antibodies detected after intraduodenal immunization

Time after respiratory infection	Serum			BAL fluid		
	Titer	A_{405} at a 1:80 dilution		Titer	A_{405} at a 1:4 dilution	
		IgG	IgA		IgG	IgA
Unimmunized mice						
Day of challenge	<40	0.004	0.002	<2	0.013	0.009
2 wk postchallenge	<40	0.005	0.000	<2	0.016	0.021
Mice immunized id with FHA[b]						
Day of challenge	3,250	0.537	0.007	88	0.080	0.000
2 wk postchallenge	4,000	0.661	0.043	112	0.427	0.147

[a] Ig anti-FHA titer is expressed as the reciprocal of the endpoint dilution of a pool from five mice and was calculated by extrapolation to zero from the linear portion of the titration curve. IgG or IgA anti-FHA was determined at a 1:80 dilution of serum or a 1:4 dilution of BAL fluid.
[b] BALB/c mice were immunized intraduodenally with 100 μg of FHA at 3 weeks before *B. pertussis* aerosol challenge.

A second advantage of local mucosal immunization is that this route may prime a population of antigen-specific B lymphocytes resident in the respiratory mucosal tissues that can be stimulated to differentiate and secrete protective antibody upon encountering the antigen associated with the whole bacterium during disease. This notion is supported by the observation of a 60-fold increase in the number of FHA-specific B lymphocytes isolated from the lungs of mice administered FHA intranasally, as determined by limiting dilution analysis (Table 4).

Mice immunized via the gut with FHA also exhibited a decreased bacterial recovery, after aerosol challenge, with a significant reduction in bacterial recovery observed in mice receiving two intraduodenal doses of FHA (Table 5). FHA is extremely susceptible to proteolysis, and significant amounts of this antigen are likely degraded in the gut. Small but detectable amounts of IgA anti-FHA as well as IgG anti-FHA were detected in BAL fluids after gut immunization and aerosol challenge, but not after aerosol challenge alone, suggesting that intraduodenal immunization may also have resulted in a primed population of FHA-specific lymphocytes in the respiratory mucosa (Table 6). Lymphocytes stimulated by antigen in the mucosal follicles of the gut can migrate via efferent lymphatics through the thoracic duct to the blood circulation; at the high endothelial venules, these circulating B lymphocytes can egress to seed distant mucosal tissues, including those of the respiratory tract (15, 44). Thus, gut immunization with FHA disseminates FHA-specific lymphocytes to the mucosal tissues of the respiratory tract where they can differentiate to secrete antibody upon a subsequent encounter with antigen.

The successful induction of protective immunity in the respiratory tract by gut immunization appears to depend on both the antigen and the pathogen analyzed. After a primary intragastric immunization of mice with Sendai virus, a secondary intranasal immunization was required to decrease virus titers in both the lower and upper respiratory tracts; neither intranasal immunization nor intraduodenal immunization alone was as protective as combined intraduodenal and intranasal immunizations (26). Multiple intragastric immunizations with *Pseudomonas aeruginosa* resulted in specific IgA antibodies detected in the gut wash and BAL fluids

of rats, but these immunized animals were not protected against intratracheal challenge with viable virulent organisms (14). Oral as well as intranasal administration of killed influenza virus elicited detectable levels of IgA but not IgG antibodies to influenza virus hemagglutinin in murine lungs, and both routes protected against lethal respiratory influenza virus challenge (5). Oral administration of vectors expressing pertussis antigens (24) or of whole killed *B. pertussis* cells (3) has been demonstrated to elicit specific antibody in mucosal secretions. We show here reproducible decreases in bacterial recoveries from the tracheas and the lungs of mice immunized either in the gut or intranasally with purified FHA in saline, in the absence of adjuvants known to enhance mucosal responses.

The follicle-associated epithelium is a highly endocytic cell layer overlying mucosal follicles, such as the bronchus-associated lymphoid tissue of the respiratory tract and the Peyer's patches of the gut, and serves as a mechanism of antigen delivery to the lymphocytes and antigen-presenting cells resident in the follicle. Antigens that can effectively bind to the follicle-associated epithelium have been shown to elicit mucosal antibody responses (7, 27, 29). FHA, one of the major adhesins of *B. pertussis*, is a filamentous protein that contains a putative lectin-binding site (8), as well as an arginine-lysine-aspartic acid (RGD) motif that mediates interactions with certain integrins (30). The lectin- and integrin-binding properties of FHA may thus contribute to its ability to persist at mucosal sites and stimulate a protective immune response.

While FHA is a major adhesin of *B. pertussis*, additional components also contribute to the adhesion of this pathogen to the ciliated epithelium of the respiratory tract (17, 19, 42). Thus, it may be necessary to combine FHA with other adhesins of *B. pertussis*, such as pertussis toxin and pertactin, to maximize protection against infection elicited by mucosal vaccines.

It is of note that specific IgG as well as IgA responses were observed after intranasal as well as intraduodenal immunization with FHA (Tables 2 and 5). IgG responses have been frequently reported after respiratory immunization (11, 22, 34, 43) and may reflect the transmission of antigen by lung macrophages through the lymphatics to the lymph nodes that drain the lung (16) as well as the transudation of IgG from the serum to the lung (37, 39). Specific IgG antibody has been demonstrated in sera and BAL fluid washes in response to oral administration of *Salmonella typhi* Ty21a to humans (13) as well as in response to oral administration of microencapsulated antigen (10) to mice. Thus, the ability of an antigen to stimulate immunity at a mucosal site may reflect intrinsic properties of the antigen, such as size, shape, and charge of the molecule, as well as lectinlike qualities and receptor binding. All of these factors may affect the interaction with antigen-presenting cells and lymphoid cells, lymphokine release, and, ultimately, the characteristics of the immune response elicited.

Of interest was the observation that total IgG and IgA antibody titers increased in the BAL fluids of unimmunized, aerosol infected mice but not in intranasally immunized mice (Table 3). At least one of the toxins associated with *B. pertussis* is known to increase capillary permeability (25); thus, this increase in total antibody in the lungs of unprotected animals may reflect leakage of total antibody from the serum into the lungs via the capillary beds. However, specific antibody to FHA, elicited by intranasal immunization prior to respiratory infection, may decrease the bacterial load in the respiratory tract, thus minimizing toxin release and capillary damage.

While the presence of specific IgG and IgA antibodies to FHA correlates with decreased bacterial recoveries in these experiments, it has not been established if protection is due to immune interference with bacterial colonization of the respiratory tract, killing of bacteria by opsonization and/or bactericidal antibody, or a combination of these mechanisms. In addition, antigen-specific T cells elicited by mucosal immunization with FHA may play a role in the observed protection against infection (9, 23).

We have thus demonstrated that mucosal administration of one of the adhesins of *B. pertussis* is effective in decreasing infection, suggesting the feasibility of an oral pertussis vaccine designed to prevent infection. Current efforts are under way to formulate delivery vehicles for pertussis antigens to protect them from proteolysis and improve their delivery to the mucosal lymphoid follicles.

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