

Bordetella pertussis Filamentous Hemagglutinin: Evaluation as a Protective Antigen and Colonization Factor in a Mouse Respiratory Infection Model

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Filamentous hemagglutinin (FHA) is a cell surface protein of *Bordetella pertussis* which functions as an adhesin for this organism. It is a component of many new acellular pertussis vaccines. The proposed role of FHA in immunity to pertussis is based on animal studies which have produced some conflicting results. To clarify this situation, we reexamined the protective activity of FHA in an adult mouse respiratory infection model. Four-week-old BALB/c mice were immunized with one or two doses of 4 or 8 μ g of FHA and then aerosol challenged with *B. pertussis* Tohama I. In control mice receiving tetanus toxoid, the CFU in the lungs increased from 10^5 immediately following infection to $>10^6$ by days 5 and 9 after challenge. Mice immunized with FHA by the intraperitoneal or intramuscular route had significantly reduced bacterial colonization in the lungs. A decrease in colonization of the trachea was also observed in FHA-immunized mice. Evaluation of antibody responses in these mice revealed high titers of immunoglobulin G (IgG) and IgM to FHA in sera and of IgG to FHA in lung lavage fluids. No IgA to FHA was detected. BALB/c mice were also passively immunized intravenously with either goat or rat antibodies to FHA and then aerosol challenged 24 h later, when anti-FHA antibodies were detected in the respiratory tract. Lung and tracheal colonization was markedly reduced in mice immunized with FHA-specific antibodies compared with those receiving control antibodies. In additional studies, the role of FHA in the colonization of the mouse respiratory tract was evaluated by using strain BP101, an FHA mutant of *B. pertussis*. FHA was important in the initial colonization of the mouse trachea, but was not required for colonization of the trachea later in the infection. FHA was not a factor in colonization of the lungs. Collectively, these experiments demonstrate (i) that systemic immunization with FHA can provide significant protection against *B. pertussis* infection in both the lower and upper respiratory tract of mice as defined by the lungs and trachea, respectively; (ii) that this protection is mediated primarily by serum antibodies to FHA, which transudate into respiratory secretions; and (iii) that FHA is an important upper respiratory tract colonization factor. These studies provide further evidence for the role of FHA in pertussis pathogenesis and immunity.

Bordetella pertussis is a bacterial respiratory tract pathogen that is the main etiologic agent of the disease pertussis, or whooping cough, in children. Although the current inactivated whole-cell vaccine has significantly reduced the incidence of pertussis (9), adverse side effects associated with the administration of this vaccine (6) have led to questions concerning its safety and continued use. As a result, efforts have focused on the development of a safer, less reactogenic pertussis vaccine.

Two components of *B. pertussis*, pertussis toxin and filamentous hemagglutinin (FHA), have received considerable attention as potential vaccine candidates and possible determinants of virulence (45). Acellular pertussis vaccines containing primarily these two pertussis antigens have been in routine use in Japan since 1981 (35), and other new acellular vaccines containing these two proteins are currently under development (29). Pertussis toxin catalyzes the ADP-ribosylation of GTP-binding proteins which are involved in signal transduction (4, 20). It is characterized by its diverse biological effects in animals, which include leukocytosis, histamine sensitization, enhanced insulin secretion, and adjuvant and mitogen activities (21, 37). FHA, a cell surface protein with a rodlike structure, appears to be

involved in the adherence of *B. pertussis* to ciliated respiratory epithelial cells (7, 27, 41-43) and to nonciliated cells (7, 27).

The interest in pertussis toxin and FHA as potential vaccine components is based mainly on animal data showing that these proteins are protective antigens. Numerous studies have demonstrated that both active and passive immunization with pertussis toxoid can protect mice against intracerebral and respiratory challenge with *B. pertussis* (23, 24, 30, 31, 34). More recently, the efficacy trial in Sweden of a single-component pertussis toxoid vaccine has shown conclusively that an immune response to pertussis toxin can provide significant protection against the disease in humans (2). In contrast, the proposed role of FHA in immunity to pertussis is based solely on animal studies which have produced some conflicting results. For example, active immunization with FHA has been reported to protect 20-day-old suckling mice from a lethal respiratory infection (23). This finding, however, could not be repeated by one of us (J. L. Cowell, unpublished observation) when using FHA with very low levels of endotoxin. Similarly inconsistent results have been obtained with passive protection tests: antibodies to FHA protected suckling mice against a lethal *B. pertussis* respiratory infection in some studies (31, 34) but not in others (23, 24).

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In the present investigation, we reexamined FHA as a protective antigen in an adult mouse respiratory infection model. The FHA used had a predominant molecular mass of 200 kilodaltons (kDa) and was low in endotoxin contamination. Following immunization, 4-week-old BALB/c mice were challenged with aerosols of *B. pertussis*; this challenge resulted in a nonlethal respiratory infection. Reductions in bacterial lung and tracheal colonization were used to assess the degree of protection. In additional experiments, we evaluated the role of FHA in the colonization of the mouse respiratory tract by using an FHA mutant of *B. pertussis* (27). We report that active immunization with FHA and passive immunization with antibodies to FHA can significantly reduce *B. pertussis* colonization in both the lungs and tracheas of mice. Furthermore, results from aerosol challenge experiments with the FHA mutant strain suggest that FHA is important for the initial colonization of the trachea of mice by *B. pertussis*.

For the purposes of this report, the trachea has been designated as part of the upper respiratory tract (19).

MATERIALS AND METHODS

Bacterial strains and growth. The Tohama phase I strain of *B. pertussis* was used for the production of FHA and as the primary respiratory challenge strain. Strain BP101, an FHA mutant, and its wild-type parental strain BP536 were used in experiments assessing the role of FHA in respiratory tract colonization. BP536 is a spontaneous nalidixic acid- and streptomycin-resistant derivative of Tohama I (27, 46). BP101, which synthesizes a truncated FHA with a molecular mass of 150 kDa, was produced by deleting an internal 2.4-kilobase *Bam*HI fragment from the FHA structural gene, *fhaB*, and returning this mutation back into the chromosome of BP536 (27). Another FHA mutant strain, BP102, was used as a negative-control strain for FHA in immunoblot experiments. BP102 is a derivative of BP536 which contains a 3.4-kilobase *Bam*HI-*Bgl*II out-of-frame deletion in *fhaB* (8). This strain is predicted to produce a 99-kDa FHA product. Although immunoblotting of BP102 with anti-FHA monoclonal antibodies demonstrates a band of this size, no reactivity is observed when specific polyclonal antiserum to FHA is used. This may be due to either a rapid degradation of this 99-kDa product or a poor polyclonal immune response to it. As such, BP102 was a suitable negative-control strain for FHA in the present studies, which involved immunoblot analysis of polyclonal antisera to FHA.

For the purification of FHA, frozen stock cultures of strain Tohama I were thawed and plated onto Bordet-Gengou (BG) blood agar plates, which were incubated for 3 days at 35°C. After passage onto fresh BG plates and incubation for 24 h, cells were harvested from the plates and inoculated into starter cultures (one plate per flask) containing 1.3 liters of Cohen-Wheeler medium supplemented with 0.1% dimethyl- β -cyclodextrin (Teijin Ltd., Tokyo, Japan). The cultures were incubated overnight at 35°C with shaking at 120 rpm. Flasks containing 1.3 liters of CL medium (15) were then inoculated with approximately 100 ml of starter culture and incubated for 24 to 48 h at 35°C with shaking. When the cultures reached their peak hemagglutination titers, they were terminated by addition of thimerosal to a final concentration of 0.02% (wt/vol) followed by an additional 2-h incubation prior to harvesting.

For aerosol infection, *B. pertussis* Tohama I was recovered from stock cultures and cultivated on BG plates as described above; strains BP101 and BP536 were grown on

BG plates containing 100 μ g of streptomycin (Sigma Chemical Co., St. Louis, Mo.) per ml. The bacterial growth on 24-h plates was harvested with 0.01 M phosphate-buffered saline (PBS) (pH 7.4) and adjusted to 140 to 150 Klett units, which corresponded to 1×10^9 to 2×10^9 CFU/ml. The bacterial suspension was kept at room temperature until it was used 1 to 2 h later for aerosol challenge.

Purification of FHA. Cultures of strain Tohama I were centrifuged at $7,000 \times g$ for 30 min, and the resulting culture supernatant was passed through a 0.2- μ m-pore-size filter (Maxi culture capsule; Gelman Sciences, Inc., Ann Arbor, Mich.). FHA was isolated from the culture supernatant by the procedure described by Sato et al. (32). Briefly, culture supernatant, following adjustment to pH 8.7, was passed through a column of spheroidal hydroxylapatite (BDH Chemicals, Poole, England). At this pH, most of the FHA was retained by the column, whereas the majority of the pertussis toxin was not. After elution of the bound FHA with a 0.1 M phosphate buffer containing 0.5 M NaCl, fetuin-Sepharose affinity chromatography was used to remove residual pertussis toxin from the sample. FHA was further purified by gel filtration on a Sepharose CL-6B column (Pharmacia, Uppsala, Sweden). Preparations of FHA were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Pertussis toxin contamination was measured by a Chinese hamster ovary (CHO) cell assay (14). Endotoxin levels were estimated by a *Limulus* amoebocyte lysate assay based on 10 endotoxin units/ng of *Escherichia coli* O113 lipopolysaccharide (LPS) and by silver staining of SDS-polyacrylamide gels (40) with purified LPS from *B. pertussis* 165 (LIST Biologicals, Campbell, Calif.) as the standard.

Preparation of antibodies to FHA. FHA (obtained from Charles R. Manclark, Center for Biologics Evaluation and Research, Food and Drug Administration) that was known to contain endotoxin was used for the production of goat anti-FHA antibodies. This FHA was purified from 4- to 5-day stationary cultures of *B. pertussis* Tohama I as described previously (32), and SDS-PAGE analysis (see Fig. 1, lane D) showed that it consisted of multiple-molecular-mass species, of which 200, 130, and 100 kDa were predominant. Endotoxin contamination was $>5\%$ (wt/wt) by the *Limulus* amoebocyte lysate assay and approximately 20% (wt/wt) by SDS-PAGE with silver staining. Before immunization, goats were bled for normal serum. The goats were immunized with 100 μ g of FHA three times, 1 month apart, by the intramuscular route. The first immunization was administered as a mixture with complete Freund adjuvant, the second was administered as a mixture with incomplete Freund adjuvant, and the third was administered without adjuvant. Goats were bled 10 days after the final immunization. The gamma globulin fraction of the sera was isolated by the method of Harboe and Ingild (13). Immunoglobulin G (IgG) was purified by DEAE-Sepharcel (Pharmacia) chromatography with 0.01 M phosphate buffer containing 0.05 M NaCl (pH 7.35) as the eluting buffer (11). The protein concentration was determined by A_{280} , using an extinction coefficient of 13.5 for pure IgG (10 mg/ml). The LPS-specific antibodies in the anti-FHA IgG preparation, as detected by enzyme-linked immunosorbent assay (ELISA), were removed by passing the preparation through an LPS-Sepharose affinity column. LPS was purified from strain Tohama I by the procedure of Johnson and Perry (18) and was coupled by using cyanogen bromide-activated Sepharose (Pharmacia).

Sprague Dawley rats (Taconic Farms, Inc., Germantown, N.Y.) were immunized with FHA which was purified at

Praxis Biologics from shake cultures of *B. pertussis* Tohama I. This FHA preparation was more homogeneous than the above FHA (see Results for analysis). Fifteen rats were immunized by the intramuscular route three times at 3-week intervals with 32 µg of FHA per immunization. In the initial immunization, FHA was mixed with complete Freund adjuvant; the remaining two were given with incomplete Freund adjuvant. For control sera, rats were immunized with PBS mixed with adjuvant. Gamma globulin was prepared from pooled sera (13). The protein concentration was determined by a modification of the method of Lowry et al. (26) with bovine serum albumin (BSA) as the standard.

Immunization of mice prior to aerosol infection. Four-week-old BALB/c mice (Taconic) were actively immunized intraperitoneally with either 4 or 8 µg of FHA (purified from shake cultures) adsorbed to aluminum hydroxide (25 µg per injection; Alhydrogel, Superfos a/c, Vedbaek, Denmark). Three weeks later, the mice were aerosol infected with *B. pertussis* Tohama I. Another group of mice was injected with a second dose of FHA without adjuvant at 3 weeks and aerosol challenged 1 week later. Control mice received one or two doses of tetanus toxoid (kindly provided by Larry Winberry, Massachusetts Public Health Biologic Laboratories, Boston). Additional groups of identically immunized mice were exsanguinated 1 day before the aerosol challenge for measurement of antibodies to FHA in sera and lung lavage fluids. In one experiment, the intramuscular and intraperitoneal routes of immunization for FHA were compared.

In passive immunization experiments, mice received either goat or rat antibodies to FHA by the intravenous route 24 h prior to aerosol infection.

Aerosol infection of mice. Aerosol infection of BALB/c mice with *B. pertussis* Tohama I was performed as described by Oda et al. (23). Aerosol challenge from a *B. pertussis* suspension of 1×10^9 to 2×10^9 CFU/ml produced a uniform, nonlethal respiratory infection in adult mice (33). Mice were sacrificed by cervical dislocation approximately 3 h after exposure (designated day 0) and on various days thereafter. The lungs and trachea (from and including the thyroid cartilage to slightly above the bifurcation) were removed and homogenized in PBS with tissue grinders. Dilutions of lung homogenates were plated on BG plates, and CFU were counted after 3 to 5 days of incubation. Tracheal homogenates were routinely plated on BG plates containing 10 or 20 µg of cephalixin (Sigma) per ml to inhibit the growth of bacterial contaminants in the trachea of some mice. In later experiments, lungs were homogenized by using a Stomacher (Tekmar Co., Cincinnati, Ohio); this method gave comparable bacterial CFU-per-lung measurements to the tissue grinders.

Aerosol infection experiments with strains BP536 and BP101 were performed exactly as described above, except that tracheal and lung homogenates were cultured on BG plates containing 100 µg of streptomycin per ml.

Lung lavage. After mice were exsanguinated by retro-orbital plexus puncture, the lungs were removed and the tracheas were cannulated with 20-gauge blunt needles. Using 1-ml syringes, lungs were washed three times with 0.3-ml volumes of ice-cold saline. Lavage fluids were centrifuged at $12,000 \times g$ for 10 min to remove cells, and final volumes were brought to 1 ml.

ELISA. Control and anti-FHA goat IgG and rat gamma globulin were evaluated for their reactivities to FHA and pertussis toxin by ELISA. Pertussis toxin was purified from culture supernatants of Tohama I by fetuin-Sepharose af-

finity chromatography. FHA and pertussis toxin were diluted in carbonate buffer (pH 9.6) to 5 µg/ml, and 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µl of the solutions per well (all incubations were performed at room temperature). The antigens were removed, and the wells were then blocked for 1 h with 200 µl of PBS (pH 7.4) containing 5% heat-inactivated fetal bovine serum. After five washes with PBS containing 0.1% Tween 20, 100 µl of goat or rat antibodies diluted in PBS with 0.3% Tween 20 and 1% fetal bovine serum were reacted for 3 h. Following five more washes, specific antibodies were detected after a 3-h incubation with a 1:2,000 dilution of rabbit anti-goat IgG peroxidase conjugate or a 1:4,000 dilution of rabbit anti-rat IgG and IgM peroxidase conjugate (Zymed Laboratories, San Francisco, Calif.) in PBS-0.1% Tween 20-1% fetal bovine serum. The plates were developed with 100 µl of *o*-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.012%) in 0.1 M citrate-phosphate buffer (pH 5.0); the reaction was stopped with 50 µl of 1 N H₂SO₄. Results are reported as the protein concentration producing an optical density of 0.2 at 490 nm calculated from a linear plot of optical density versus protein concentration.

Class-specific antibody responses to FHA in mice were evaluated by using the procedure described above, except that biotinylated goat anti-mouse IgG or IgA and rabbit anti-mouse IgM antibody probes (Zymed) were used at a 1:10,000 dilution. The antibodies were then reacted with a 1:8,000 dilution of avidin-peroxidase (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). Titers are reported as the reciprocal of the dilution giving an optical density 5 times that of the pooled normal mouse serum control for serum samples and 5 times that of the conjugate control for lung lavage fluids.

Goat IgG and rat gamma globulin were also assessed for their reactivity to *B. pertussis* LPS by a modified ELISA. For improved detection of antibodies to LPS, MgCl₂ was used in the procedure as described by Ito et al. (17). LPS was isolated from Tohama I cells as described previously (18). Plates were coated overnight with (per well) 1 µg of LPS diluted in saline with 0.02 M MgCl₂. The plates were then washed three times with the saline-MgCl₂ solution and blocked for 1 h with the same solution containing 1% BSA. Antibodies were diluted in saline-MgCl₂-BSA and reacted for 3 h. Anti-LPS antibodies were detected by using the peroxidase conjugates described above.

Immunoblotting. Goat and rat antibodies to FHA were also evaluated for specificity by immunoblot analysis with whole-cell lysates of *B. pertussis* as the antigen. *B. pertussis* Tohama I and BP102, which was used as the negative control strain for FHA, were grown overnight in CL medium at 35°C with shaking. The cell pellet from 10 ml of culture was suspended in 3 ml of SDS-PAGE sample buffer containing 2% SDS, and then the cell lysate was reduced with 5% 2-mercaptoethanol and boiled for 5 min. Whole-cell lysates (10 to 15 µl) were resolved by SDS-PAGE in a 10% polyacrylamide gel and transferred to nitrocellulose membranes (39). The blots were blocked for 1 h in PBS containing 1% BSA and then reacted with 5 µg of goat or rat antibodies per ml in 20 ml of the PBS-BSA buffer for 1 h. The blots were washed with PBS containing 0.1% Tween 20; bound antibodies were detected by using the peroxidase-conjugated anti-goat and anti-rat antibody probes described above. The blots were developed in a solution of 4-chloro-1-naphthol (0.5 mg/ml; Sigma) plus hydrogen peroxide (0.01%).

Statistical analysis. Unless otherwise stated, data were tested for statistical significance by Student's *t* test.

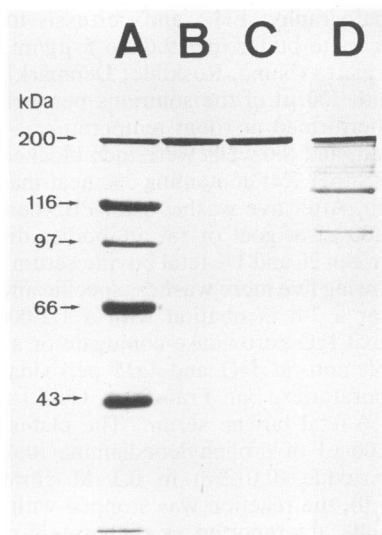


FIG. 1. SDS-PAGE analysis of FHA preparations. Each sample contained 10 μ g of protein which was resolved in a 7.5% polyacrylamide gel and visualized by staining with Coomassie blue. Lanes: A, molecular mass standards (myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; BSA, 66 kDa; ovalbumin, 43 kDa); B and C, two different FHA preparations isolated from shake cultures of *B. pertussis* Tohama I; D, FHA preparation isolated from 4- to 5-day stationary cultures of *B. pertussis* Tohama I (32). FHA was purified from culture supernatants by using the procedure described by Sato et al. (32).

RESULTS

FHA characterization. SDS-PAGE analysis of FHA preparations is shown in Fig. 1. Two FHA preparations, which were used for most of the experiments, were isolated from shake cultures of *B. pertussis* Tohama I and had a predominant polypeptide species with a molecular mass of 200 kDa (Fig. 1, lanes B and C). Fetuin-Sepharose affinity chromatography reduced pertussis toxin contamination to less than 0.005% (wt/wt) as determined by the CHO cell assay. The endotoxin level in these preparations was approximately 0.05% (wt/wt) by the *Limulus* amoebocyte lysate assay and by SDS-PAGE with silver staining. Another preparation of FHA (obtained from C. R. Manclark) was used for the production of goat antibodies. SDS-PAGE analysis of this FHA, which was purified from 4- to 5-day stationary cultures of Tohama I, revealed several polypeptides with molecular masses ranging from 100 to 200 kDa (Fig. 1, lane D). If the faster-migrating components are proteolytic breakdown products of the 200-kDa protein, as some investigators have suggested (16), these findings indicate that more proteolysis of FHA occurs in stationary than in shake cultures of *B. pertussis* Tohama I. Storage was not a factor, since all FHA preparations were kept at -70°C .

Active immunization experiments. Lung colonization of BALB/c mice immunized intraperitoneally with one dose of 4 or 8 μ g of FHA and challenged with *B. pertussis* Tohama I 3 weeks later is shown in Fig. 2A. In control mice receiving 8 μ g of tetanus toxoid, bacterial number in the lungs increased from 10^5 CFU immediately following aerosol infection to 3×10^6 CFU by days 5 and 9 after challenge. Immunization with either dose of FHA reduced this colonization, but still permitted bacterial multiplication to occur from the initial inoculum. Another group of mice received a second dose of FHA 3 weeks after the first injection and

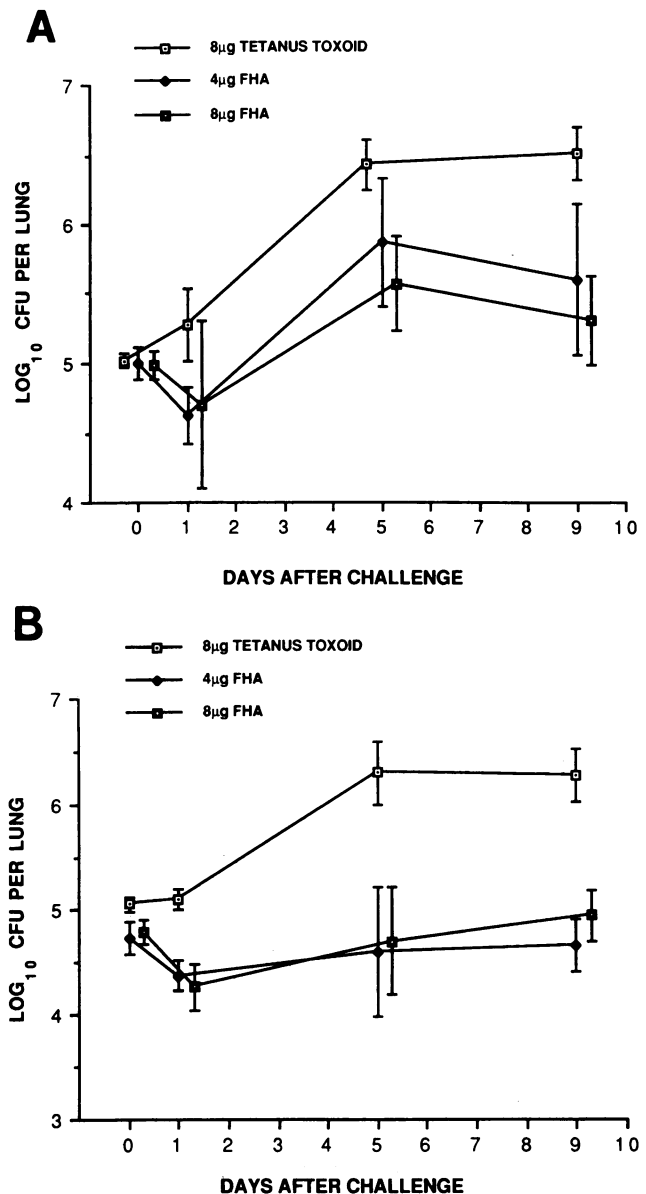


FIG. 2. *B. pertussis* colonization of the lungs of adult BALB/c mice actively immunized with FHA. Mice were immunized intraperitoneally with 4 or 8 μ g of FHA adsorbed to aluminum hydroxide and then aerosol challenged with *B. pertussis* Tohama I 3 weeks later (A). Another group of mice were given a second dose of FHA without adjuvant at 3 weeks and challenged 1 week later (B). Control mice received tetanus toxoid. The plots show the geometric mean \pm standard deviation (bars) for five mice per time point.

was aerosol infected 1 week later. Lung CFU in these mice was significantly reduced compared with the controls and never increased to a level above the initial challenge level (Fig. 2B, $P < 0.01$ for FHA-immunized mice versus controls at all time points). There was no difference in the level of protection conferred by two doses of 4 or 8 μ g of FHA.

Additional groups of identically immunized mice were evaluated for their antibody responses to FHA by ELISA (Table 1). A single 4 or 8- μ g dose of FHA elicited a detectable IgG response in both sera and lung lavage fluids. In mice receiving a second injection of FHA, IgG levels in sera and lung lavage fluids were much higher, correlating

TABLE 1. Antibody response of BALB/c mice immunized with FHA^a

FHA dose (μ g/mouse)	Serum antibody titer ^b						Lung lavage fluid titer ^b					
	First injection			Second injection			First injection			Second injection		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
4	25,600	<50	<50	819,200	3,200	<50	8	<2	<2	512	<2	<2
8	51,200	<50	<50	819,200	6,400	<50	32	<2	<2	1,024	<2	<2
Control ^c	<50	<50	<50	<50	<50	<50	<2	<2	<2	<2	<2	<2

^a Mice were immunized intraperitoneally with FHA mixed with 25 μ g of aluminum hydroxide and then bled and lung lavaged 3 weeks later. Another group of mice received a second injection of FHA without adjuvant at 3 weeks and were bled and lung lavaged 1 week later. Sera and lung lavage fluids were pooled for four to six mice per group. Class-specific antibodies to FHA were measured by ELISA.

^b Reciprocal of the dilution giving an optical density (490 nm) 5 times that of the normal mouse serum control for serum samples or 5 times that of the conjugate control for lung lavage fluid samples.

^c Control dose was 8 μ g of tetanus toxoid per mouse.

with the increased protection observed in these mice. IgM antibodies to FHA were found only in the sera of mice receiving two doses of FHA; no IgA to FHA was detected.

Because intramuscular injection has been the traditional route for pertussis vaccination, we believed that it was important to evaluate the immunogenicity and protective activity of FHA given by this route. Two 8- μ g doses of FHA administered to mice by the intramuscular route induced anti-FHA IgG titers in sera and lung lavage fluids (data not shown) equivalent to those obtained by intraperitoneal injection (Table 1). IgM and IgA to FHA were not detected. Consistent with the IgG antibody response, the intramuscular and intraperitoneal injection routes afforded similar levels of protection against *B. pertussis* infection in the lungs (Fig. 3A). By day 14, no differences between immune and control groups were observed, as a result of a marked reduction in the bacterial lung counts of control mice at this time point. This was a consistent finding throughout the study.

Colonization of the trachea was also evaluated in this experiment. It is generally thought that in the natural disease, *B. pertussis* infection remains localized to the ciliated epithelium of the human respiratory tract. Therefore, we believed that *B. pertussis* colonization of the ciliated epithelium of the mouse trachea, as opposed to the lung, would more closely reflect host-parasite interactions that occur in the human disease. Colonization of the tracheal ciliated epithelium of mice by *B. pertussis* has been reported previously (33). Tracheal colonization in mice aerosol challenged with *B. pertussis* Tohama I is shown in Fig. 3B. Similar to colonization of the lungs, *B. pertussis* cells in the trachea multiplied and reached a maximum level at days 5 and 9. Compared with tetanus toxoid-immunized controls, FHA-immunized mice had significantly lower levels of tracheal colonization at days 0 and 1 ($P < 0.01$, except for mice immunized intramuscularly with FHA at day 0, for which $P < 0.05$) and again at days 5 and 9 postchallenge ($P < 0.01$). These differences, however, were not as great as those observed in the lungs (Fig. 3A).

Passive immunization experiments. For passive immunization experiments, antibodies to FHA were produced in goats and rats. Preimmune and immune goat IgG and control and immune rat gamma globulin were prepared as described in Materials and Methods. Reactivities to FHA, pertussis toxin, and LPS were evaluated by ELISA, and these data are shown in Table 2. The preimmune goat IgG showed some slight reactivity to FHA and no reactivity to pertussis toxin or LPS. The anti-FHA goat IgG produced a positive reaction at 7.3 ng/ml to FHA; the preparation also had reactivity with LPS and minimal reactivity with pertussis toxin. The antibodies to LPS were removed by passing the IgG through an

LPS-Sepharose affinity column; this reduced the LPS reactivity to preimmune levels. Control rat gamma globulin had no detectable reactivity to any of the antigens. The immune rat gamma globulin to FHA gave a positive reaction at 54 ng/ml to FHA, with no detectable reactivity to pertussis toxin or LPS.

The specificity of the goat and rat anti-FHA antibodies was further evaluated against whole-cell lysates of *B. pertussis* by immunoblotting. Preimmune goat IgG and control rat gamma globulin had no reactivity to whole-cell lysates of Tohama I or BP102, a negative-control strain for FHA derived from Tohama I (Fig. 4, lanes 1 and 2). The goat (LPS-adsorbed) and rat anti-FHA antibodies, against Tohama I whole-cell lysates, reacted predominantly with bands that migrated at 200 kDa; reactivity was also observed to some minor, faster-migrating bands (Fig. 4, lanes 3). The total lack of reactivity of these antisera with lysates of strain BP102 confirmed their specificity for FHA (Fig. 4, lanes 4).

Adult BALB/c mice were passively immunized intravenously with the antibody preparations 24 h prior to aerosol challenge with *B. pertussis*. On the basis of our studies (data not shown) and those of others (28, 38; R. Shahin, personal communication), this period allows a maximum amount of antibody to transudate from the serum into the respiratory tract. In mice injected with 250 or 500 μ g of goat anti-FHA IgG, *B. pertussis* colonization of the lungs was significantly reduced at days 1 through 9 compared with that in control animals receiving preimmune IgG (Fig. 5; $P < 0.01$, except for mice receiving the 500- μ g dose at day 1, for which $P < 0.05$). There appeared to be some dose-dependent protection at day 5 ($P < 0.02$ for mice receiving 500 μ g of anti-FHA IgG compared with those receiving 250 μ g). Rat gamma globulin to FHA (1 mg) also afforded protection against *B. pertussis* respiratory infection. Bacterial multiplication was inhibited in the lungs and tracheas of mice administered rat anti-FHA antibodies; this resulted in a marked reduction in colonization at these sites compared with controls (Fig. 6; $P < 0.01$ for days 1 through 9).

Colonization experiments with strain BP101. Strain BP101, a mutant that synthesizes a truncated FHA with a molecular mass of 150 kDa, has been shown to adhere poorly to CHO cells and ciliated rabbit epithelial cells in vitro (27). Colonization of the respiratory tract by this FHA mutant was compared with colonization by its wild-type parental strain BP536. No difference in the ability to colonize the lungs was found between these two strains (Fig. 7). In contrast, colonization of the trachea by BP101 was very different from colonization by BP536. After aerosol challenge, BP536 was able to colonize the tracheas of mice throughout the 10-day test period (Table 3). BP101, however, had lower levels of

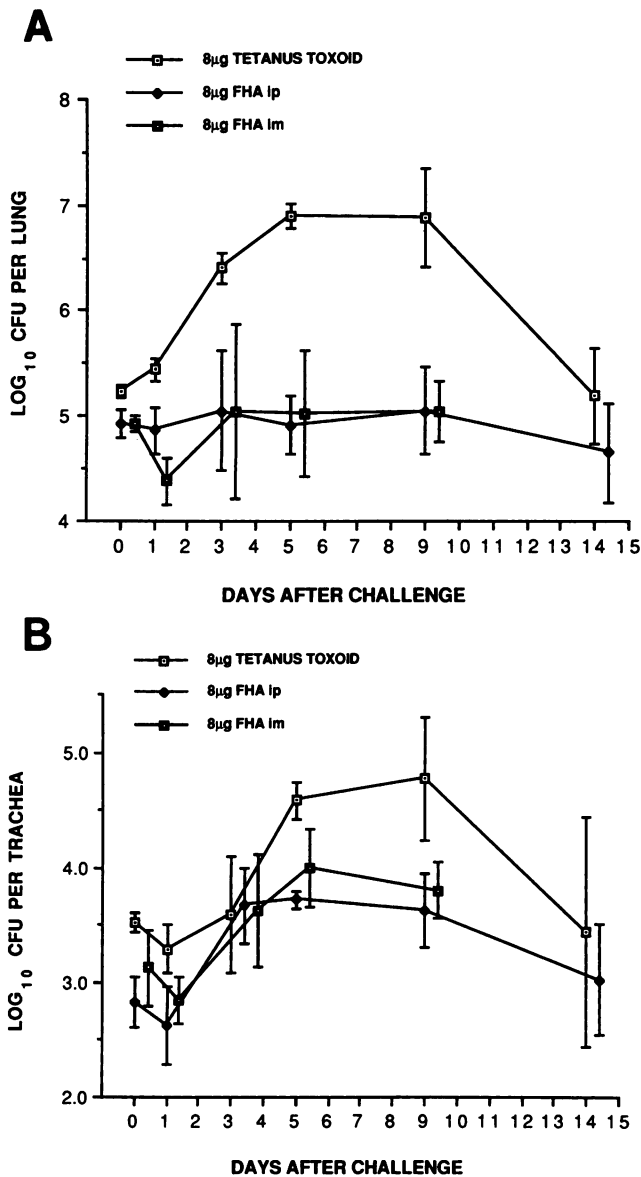


FIG. 3. *B. pertussis* colonization of the lungs (A) and tracheas (B) of BALB/c mice immunized with two 8- μ g doses of FHA administered by the intraperitoneal or intramuscular route. Mice were initially immunized with FHA adsorbed to aluminum hydroxide, given a second injection without adjuvant 3 weeks later, and aerosol challenged 1 week after the second injection. Control mice received tetanus toxoid by the intraperitoneal route. The plots show the geometric mean \pm standard deviation (bars) for five mice per time point.

colonization immediately following challenge at day 0 (Table 3). Moreover, on days 1 and 5 for the first experiment and on day 1 for the second experiment, BP101 was not detected in the tracheas of the majority of mice. At day 1 (pooled data from both experiments), only 6 of 15 mouse tracheas were colonized with BP101, compared with 15 of 15 colonized with BP536; at day 5 in the first experiment, one of five mouse tracheas were colonized with BP101, whereas all five mice challenged with BP536 were colonized in the trachea. Unexpectedly, BP101 was able to recolonize the tracheas of mice by day 10 in the first experiment and by day 5 in the second experiment. *B. pertussis* isolated from tracheas at

TABLE 2. Characterization of goat IgG and rat gamma globulin to FHA by ELISA

Immunoglobulin	Antibody concn (μ g/ml) producing optical density at 490 nm of 0.2 with coating antigen:		
	FHA	Pertussis toxin	LPS
Goat IgG ^a			
Preimmune	14.4	>200	>200
Immune	7.3×10^{-3}	82.5	486.1×10^{-3}
Immune-LPS adsorbed	8.4×10^{-3}	178.7	>200
Rat gamma globulin ^b			
Control	>200	>200	>200
Immune	54.0×10^{-3}	>200	>200

^a Immunization of goats and isolation of IgG from serum were performed as described in Materials and Methods.

^b Immunization of rats and isolation of gamma globulin were performed as described in Materials and Methods.

days 5 and 10 after challenge still retained the mutant FHA phenotype as determined by immunoblot analysis of whole-cell lysates with anti-FHA goat IgG as the probe (data not shown).

DISCUSSION

The findings presented here demonstrate that FHA is a protective antigen in adult mice that were aerosol infected with *B. pertussis*. FHA active immunization elicited high titers of anti-FHA IgG, which were detected in both sera and lung lavage fluids of mice. This antibody response correlated with a significant reduction in lung and tracheal colonization by *B. pertussis*. A similar level of protection against respiratory tract colonization was achieved by passive immunization of mice with FHA-specific antibodies. In addition, experiments with strain BP101, an FHA mutant, showed that intact FHA was essential for initial colonization of the tracheas of mice, but was not required for tracheal colonization later in the infection. FHA was found not to be a factor in colonization of the lungs. These studies provide evidence for the role of FHA in the pathogenesis of *B. pertussis* infection. Furthermore, they clearly establish FHA as a protective antigen and potential vaccine component.

Our data showing that active immunization with FHA can induce significant protection against *B. pertussis* lung infection confirm results obtained in other studies. Oda et al. (23) reported that FHA immunization protected suckling mice against a lethal aerosol challenge, whereas Robinson et al. (30) found that immunization with FHA markedly reduced lung colonization in adult mice infected intranasally. Colonization of the lungs, however, may not fully reflect the host-parasite interactions that occur in the natural disease, in which *B. pertussis* infection is believed to be limited to the ciliated epithelium of the respiratory tract. Therefore, we believed that it was important to evaluate the colonization of the respiratory ciliated epithelium of the mouse, specifically the trachea. The present study is the first to document protective activity against tracheal colonization in mice immunized with FHA. Collectively, our results demonstrate that FHA immunization can provide significant protection against *B. pertussis* infection in both the lower and upper respiratory tract of mice as defined by the lungs and trachea, respectively.

Our interest in reexamining FHA as a protective immunogen arose from the observation that FHA with low levels of

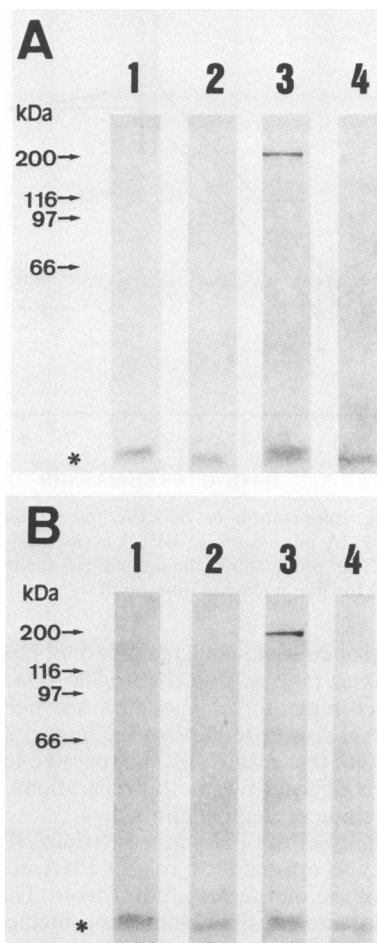


FIG. 4. Immunoblot analysis of goat and rat anti-FHA antibodies. Whole-cell lysates of *B. pertussis* Tohama I and BP102, a negative-control strain for FHA, were prepared as described in Materials and Methods. These samples were resolved by SDS-PAGE in a 10% polyacrylamide gel and transferred to nitrocellulose membranes (39). The blots were incubated with the goat or rat anti-FHA antibodies ($5 \mu\text{g/ml}$ in a total volume of 20 ml) and then reacted with peroxidase-conjugated antibody probes. Lanes 1 and 3 contain whole-cell lysates of Tohama I as antigen; lanes 2 and 4 contain whole-cell lysates of BP102. (A) Lanes 1 and 2, blots reacted with preimmune goat IgG; lanes 3 and 4, blots reacted with anti-FHA goat IgG (LPS adsorbed). (B) Lanes 1 and 2, blots reacted with control rat gamma globulin; lanes 3 and 4, blots reacted with anti-FHA rat gamma globulin. The asterisk indicates the dye front.

endotoxin no longer protected 20-day-old suckling mice from a lethal respiratory infection (Cowell, unpublished). In our studies, FHA with relatively low levels of endotoxin was very immunogenic in adult mice and induced consistent protection in the respiratory tract. Because FHA is poorly immunogenic in mice less than 20 days of age (23), one likely explanation for the conflicting results is that a small amount of contaminating endotoxin is required for enhancing the immunogenicity and protective activity of FHA in very young mice. Another possibility is that *B. pertussis* endotoxin itself is inducing either specific or nonspecific protection. In regard to the latter possibility, *B. pertussis* endotoxin has been shown to protect animals nonspecifically against a variety of pathogens (3). Further investigation is needed to define the function of endotoxin in FHA protection of young mice.

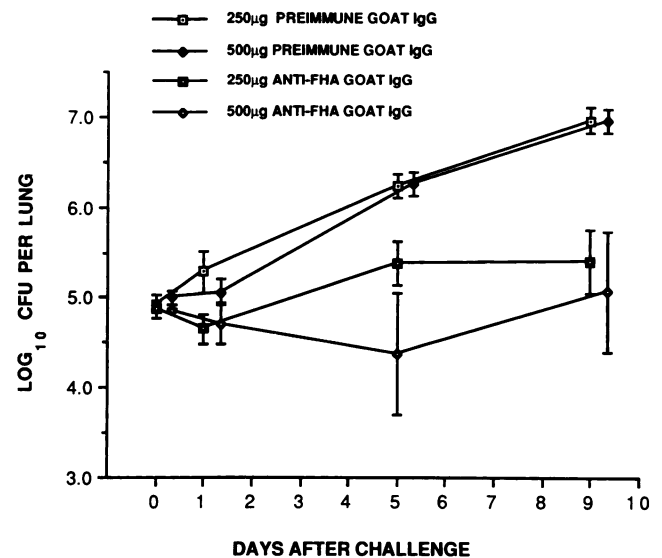


FIG. 5. Lung colonization in mice passively immunized with 250 or 500 μg of either preimmune or anti-FHA goat IgG. The mice were given the antibody intravenously 24 h prior to aerosol challenge with *B. pertussis*. The plots show the geometric mean \pm standard deviation (bars) for five mice per time point.

Following active immunization of mice with FHA, IgG to FHA was detected in both sera and lung lavage fluids. The latter was an important finding because it showed that antibody to FHA was present in the respiratory tract, the actual site of *B. pertussis* challenge and subsequent colonization. To determine the role of this anti-FHA antibody in protection, we performed passive-immunization experiments. Goat or rat antibodies to FHA were injected intravenously into mice 24 h prior to aerosol challenge, a period which allowed the antibodies to passively diffuse from the serum into the respiratory tract. *B. pertussis* lung and tracheal colonization was significantly reduced in animals receiving FHA-specific antibodies; this level of protection was comparable to that obtained by active immunization. These results indicate that pulmonary protection following systemic FHA immunization is mediated primarily, if not totally, by a serum antibody response that transudates into the respiratory tract. This finding has important implications for vaccine development because it suggests that FHA-mediated protection in the respiratory tract can be correlated to anti-FHA antibody levels in serum. This does not rule out a possible role for cellular immunity to FHA (8, 10), nor does it diminish the potential importance of a mucosal antibody response to FHA in the respiratory tract (12). At this time, however, very little is known about the contribution of these immune mechanisms to protection by FHA and other protective antigens of *B. pertussis*. Before the most efficacious pertussis vaccines can be developed, these types of questions must be addressed.

In previous investigations, passive immunization experiments have produced conflicting results. Sato et al. (34) reported that rabbit anti-FHA gamma globulin reduced lung colonization and prevented leukocytosis and death in suckling mice that were aerosol challenged with *B. pertussis*. In another paper, Sato and Sato (31) showed that mouse gamma globulin and goat IgG to FHA also protected suckling mice against a lethal aerosol challenge. In contrast, two studies published by Oda et al. (23, 24) revealed that goat IgG, a mouse monoclonal antibody, and human colostral antibody

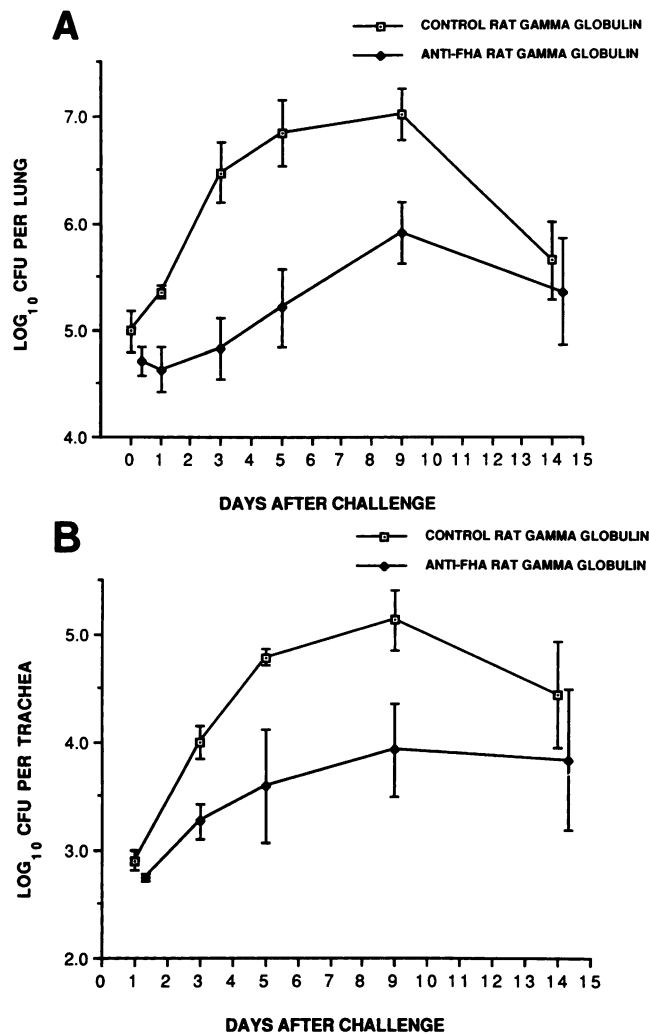


FIG. 6. Colonization of the lungs (A) and tracheas (B) of mice passively immunized with 1 mg of control or anti-FHA rat gamma globulin. Antibody was administered intravenously 24 h prior to aerosol challenge with *B. pertussis*. The plots show the geometric mean \pm standard deviation (bars) for five mice per time point.

to FHA conferred little or no protection in the same mouse model. It is difficult to explain why protection was obtained in some studies but not in others. The difference in the antibodies to FHA may be one simple explanation. Alternatively, the inconsistencies may be related to the route of antibody administration and subsequent time of aerosol challenge. In all of the above-cited studies, antibodies were injected intraperitoneally 30 min to 2 h before infection. In some cases, this procedure may not have allowed a sufficient amount of antibody to reach the lungs by the time of bacterial challenge. This rationale, along with data showing that antibodies injected intravenously could be detected in the lungs 24 h later, formed the basis for our protocol of challenging with *B. pertussis* 24 h after intravenous administration of FHA-specific antibodies.

It is also worth noting that goat and rat antibodies to FHA conferred similar levels of protective activity, although they were raised against FHA preparations with very different profiles in SDS-PAGE. Rats were immunized with relatively intact FHA, which had a predominant molecular mass of 200 kDa (Fig. 1, lanes B and C), whereas goat IgG was raised

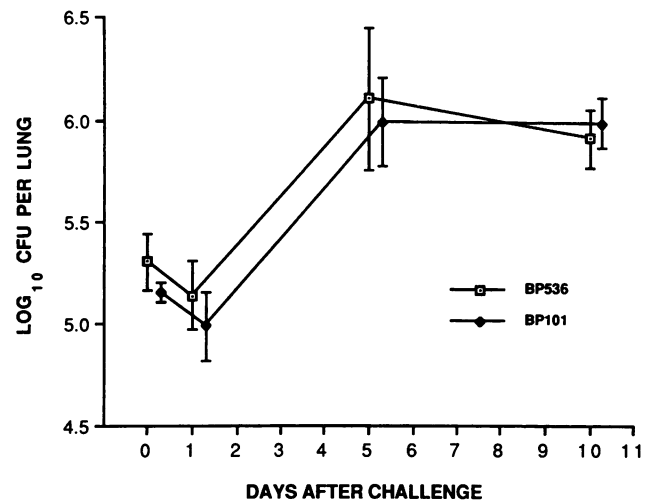


FIG. 7. Lung colonization in BALB/c mice following aerosol challenge with FHA mutant strain BP101 or its wild-type parental strain BP536. The plots show the geometric mean \pm standard deviation (bars) for five mice per time point.

against FHA that consisted of a number of polypeptides with molecular masses ranging from 100 to 200 kDa (Fig. 1, lane D). The faster-migrating components are believed to be proteolytic breakdown products of the larger, 200-kDa protein (16). From the results of the passive-immunization experiments, it appears that both preparations of FHA are capable of eliciting protective antibodies.

The availability of the FHA mutant strain BP101 gave us an opportunity to evaluate the role of FHA in *B. pertussis* colonization of the mouse respiratory tract. That FHA was found to be a factor in the colonization of the tracheas of mice, but not their lungs, was not totally unexpected. There are good data, both in vitro and in vivo in animal models, supporting the role of FHA in *B. pertussis* adherence to ciliated epithelial cells of the respiratory tract (27, 41–43). Furthermore, there is evidence from several studies indicating that FHA is not essential for colonization of the lungs. Weiss et al. (47) found that a Tn5-induced mutant of *B. pertussis*, deficient in FHA, was not significantly altered in its 50% lethal dose in infant mice by intranasal challenge (compared with the wild type) and could be recovered from the lungs of the dead animals. This mutant, however, does produce small amounts of wild-type FHA. Tuomanen et al. (41, 43) reported that the same FHA-deficient mutant failed to colonize the upper respiratory tract of rabbits and, instead, passed into the alveoli, where it persisted and produced lung pathology. These data, along with our results, suggest that FHA is not a crucial adhesin in the lower respiratory tracts of mice.

The ability of the FHA mutant BP101 to recolonize the trachea, however, was surprising. After being cleared to undetectable levels in the tracheas of the majority of mice early in the infection, BP101 was able to recolonize the tracheas at a later time either from the few undetected bacteria remaining (the limit of detection was 20 CFU) or from the large number of bacteria in the lungs. The latter would suggest that mouse lungs can act as a reservoir from which *B. pertussis* can reinfest the upper airways. These results indicate that FHA is important for initial adherence and colonization of the trachea, but that other host-parasite interactions occur later in the infection which allow BP101 to recolonize the trachea. These new interactions may be due

TABLE 3. Colonization of the trachea by FHA mutant strain BP101 and its wild-type parental strain BP536 following aerosol challenge

Strain and expt no.	Log ₁₀ CFU/trachea ^a (no. of animals colonized/total no. of animals) at postchallenge day:			
	0	1	5	10
BP536				
1	3.94 ± 0.12 (5/5)	2.68 ± 0.21 (5/5)	2.52 ± 0.71 (5/5)	4.02 ± 0.31 (5/5)
2	4.02 ± 0.15 (10/10)	3.25 ± 0.12 (10/10)	2.54 ± 0.69 (10/10)	4.48 ± 0.38 (10/10)
BP101				
1	3.16 ± 0.24 ^b (5/5)	0.58 ± 0.80 (2/5) ^c	0.52 ± 1.16 (1/5) ^d	2.79 ± 0.71 (5/5)
2	3.29 ± 0.21 ^b (10/10)	0.60 ± 0.80 (4/10) ^c	3.13 ± 0.43 (9/9)	3.58 ± 0.59 (10/10)

^a Geometric mean ± standard deviation. The limit of detection was 20 CFU per trachea. For calculation of the geometric mean, animals with no detectable CFU were arbitrarily assigned 1 CFU.

^b $P < 0.01$ compared to BP536 for each experiment.

^c $P < 0.01$ by chi-square analysis for pooled data from both experiments at day 1 (6 of 15 mice colonized with BP101 versus 15 of 15 mice colonized with BP536).

^d $P < 0.05$ compared with BP536 in experiment 1 by Fisher's exact test.

to a modification of the infecting organism in vivo which alters its adherence capabilities. Another explanation is that early in the respiratory infection, when mucociliary clearance is still effective, FHA may be necessary for adherence to the ciliated epithelium. Later in the infection, however, when ciliated epithelial-cell injury occurs resulting in ciliostasis, as suggested by organ culture studies (25), *B. pertussis* may be able to adhere and colonize the upper airways even in the absence of FHA.

The mechanism(s) of anti-FHA antibody-mediated protection in the respiratory tract remains undefined. Because FHA is believed to be a primary adhesin, one likely function of FHA-directed antibodies in the upper respiratory tract may be in blocking adherence. Both the IgG and IgA classes of antibody elicited after natural disease have been shown to possess antiadherence activity (44). In view of the data in the present study which demonstrate that FHA is not essential for colonization of and presumably adherence in the lungs, anti-FHA antibodies must possess other additional effector functions. Antibodies to FHA may promote extracellular killing with complement or, as opsonins, mediate intracellular killing by phagocytes. There is evidence that antibodies to *B. pertussis* are bactericidal via the complement pathway (1). In addition, Muse et al. showed that specific antiserum does facilitate phagocytosis and killing of *B. pertussis* by guinea pig alveolar macrophages in vitro (22). These data appear to contradict results from other studies, however, which suggest that *B. pertussis* can survive and multiply within phagocytic cells (5; R. L. Friedman and P. Z. Detskey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, D128, p. 103). Our research efforts are now focused on defining the mechanism(s) by which anti-FHA antibodies confer protection in the respiratory tract.

The nonlethal respiratory infection of adult mice with *B. pertussis* is a model for colonization, not for disease. Adult mice do not generally develop clinical signs of *B. pertussis* infection, such as leukocytosis, reduced body weight gain, and death, as do younger mice (33). Thus, protection against disease following FHA immunization can only be inferred from reductions in bacterial lung and tracheal colonization in the adult mouse model. Evaluation of potential protective antigens by measuring reductions in pulmonary colonization, however, does have merit. Indeed, protection against lung colonization in mice following a sublethal respiratory challenge has some correlation with vaccine efficacy in humans (36). Moreover, with the quantitation of tracheal colonization, the adult mouse respiratory infection model may now be a more relevant model for human infection.

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