INFECTION AND IMMUNITY, Apr. 1993, p. 1447–1452 Vol. 61, No. 4
0019-9567/93/041447-06$02.00/0
Copyright © 1993, American Society for Microbiology

Long-Lived Respiratory Immune Response to Filamentous Hemagglutinin following *Bordetella pertussis* Infection

DIANA F. AMSBAUGH,* ZHONG-MING LI, AND ROBERTA D. SHAHIN

Laboratory of Pertussis, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892-0029

Received 15 July 1992/Accepted 11 January 1993

Systemic and mucosal B-cell-mediated immune responses to purified filamentous hemagglutinin (FHA) in mice were analyzed at different times following a single respiratory infection with *Bordetella pertussis*. Serum immunoglobulin G (IgG) anti-FHA and respiratory IgG and IgA anti-FHA antibodies were first detected at 3 weeks postinfection, reached high levels by 8 weeks postinfection, and remained at high levels 12 to 32 weeks postinfection. FHA-specific B lymphocytes isolated from the spleens or lungs of uninfected control mice or mice convalescing from *B. pertussis* respiratory infection were analyzed in limiting-dilution cultures. Analysis of culture supernatants for the production of antibodies to FHA revealed an increased frequency of FHA-specific B cells of both the IgG- and the IgA-secreting classes in the lungs and tracheas of aerosol-challenged mice; these levels remained high as late as 25 weeks postinfection, compared with those in uninfected controls. No corresponding increase in the frequency of FHA-specific B cells in the spleens of aerosol-infected mice was observed. This long-lasting response observed in cultured cells was radiation resistant, a result suggesting that this response was due to B cells already activated in vivo. Polymerase chain reaction analysis revealed low but detectable levels of *B. pertussis* chromosomal DNA in 75% of mice tested at 8 weeks postinfection and 37.5% of mice tested at 26 weeks postinfection, at which times high levels of anti-FHA antibody were detected. One explanation for these data may be that, in this animal model, a major adhesin of *B. pertussis* can persist and interact with components of the immune system to stimulate the production of specific antibody in the respiratory tract many weeks after a single *B. pertussis* infection.

Bordetella pertussis, the etiologic agent of pertussis (whooping cough), is a gram-negative rod that infects as an aerosol and colonizes the ciliated epithelium of the respiratory tract. *B. pertussis* is one of the most infectious bacterial pathogens known, with a household attack rate of up to 83% (20, 24). Morbidity and mortality from pertussis are highest in infants; in adults and older children, pertussis often is a milder disease, of low mortality, and is at times clinically noncharacteristic (5).

Parenteral vaccination with whole-cell pertussis vaccine at 2, 4, 6, and 18 months of age, with a fifth and final dose at 4 to 6 years, has been successful in controlling the incidence of pertussis in children in the United States (5); however, vaccine-induced immunity to pertussis appears to wane in young adulthood (9, 15). In contrast, infection with *B. pertussis* appears to stimulate long-lived immunity to reinfection (5). Following natural *B. pertussis* infection, high titers of immunoglobulin A (IgA) antibodies (Abs) to pertussis antigens (Ags) are detected in the nasal secretions of convalescing patients (20, 37); in healthy parenterally vaccinated 11-month-old infants, with no history of pertussis, titers of nasal IgA Abs to pertussis Ags are significantly lower (37).

Long-lived immunity to pertussis may reflect the induction of a memory immune response at the respiratory mucosa by natural infection but not by parenteral immunization. *B. pertussis* has been shown to be associated with the ciliated epithelium of the trachea and bronchial tree of mice following experimental respiratory infection (28, 30). The purpose of this study was to characterize the respiratory and systemic immune response to Ags of *B. pertussis* in mice following respiratory infection.

* Corresponding author.

MATERIALS AND METHODS

Mice. Specific-pathogen-free (C57BL/6 × C3H/HeN)F_1 (B6C3) females were obtained at 5 weeks of age from either the Animal Production Program, Division of Cancer Research Treatment, National Cancer Institute, Frederick, Md., or Charles River, Portage, Mich. The mice were maintained in microisolators under specific-pathogen-free conditions.

Ags. Filamentous hemagglutinin (FHA) was kindly provided by Jean Petre, Smith Kline Biologicals, Rixensart, Belgium, and Alan Kimura, Praxis Biologics, Inc., Rochester, N.Y. Preparations of FHA used in these studies ran predominantly as a single band of 220 kDa on sodium dodecyl sulfate-polyacrylamide gels and had less than 0.005% pertussis toxin (PT) contamination. These preparations contained approximately 0.05% endotoxin (14) or 0.0006% endotoxin (Smith Kline preparation). Lipooligosaccharide (LOS) was obtained from List Biological Laboratories, Inc., Campbell, Calif.; PT and pertactin were obtained from Smith Kline; and type 2 and 3 fimbriae were obtained from Connaught Laboratories, Ltd., Willowdale, Ontario, Canada.

Aerosol challenge. A 21-h culture of *B. pertussis* 18323 was suspended in a solution of sterile phosphate-buffered saline (PBS) at a concentration of approximately 10^9 CFU/ml. This inoculum was administered to mice as an aerosol by use of a standard nebulizer (Fisons Corp., Bedford, Mass.) within a biosafety level 3 glove box (Blickman Co., Weehawken, N.J.) as previously described (33). Mice were removed from the chamber 1 h after termination of the aerosol challenge, at

which time viable *B. pertussis* cells cannot be cultured from the surface of the animals or the chamber (30). For each aerosol challenge performed, two mice were sacrificed upon 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 1 h after aerosol challenge. Age-matched control mice were uninoculated and remained under specific-pathogen-free conditions for the duration of the experiments.

Analysis of respiratory tract and serum immunoglobulin (Ig). Serum, bronchoalveolar lavage (BAL) fluids, and nasal washes (NW) were collected from anesthetized mice as previously described (32). All fluids were frozen at −20°C until analysis by an enzyme-linked immunosorbent assay (ELISA).

Isolation of lymphocytes. Mice anesthetized with an intraperitoneal injection of 0.4 ml of a 2% solution of 2,2,2-tribromoethanol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) were bled from the brachial artery. The heart was perfused with 3 to 6 ml of sterile Alsever's solution until the lungs visibly lightened; lungs and tracheas were removed aseptically as a unit. After being minced with scissors, pools of lungs and tracheas from four to six mice were digested in the neutral protease dispase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 2 h with constant stirring at 35°C (29). The dispersed cells were filtered through Nitex (Tetko Inc., New York, N.Y.), washed, and treated with ACK lysing buffer (B & B Research Laboratories, Inc., Fiskeville, R.I.) to remove erythrocytes. Spleens were removed aseptically, teased through a screen, washed, and treated with ACK lysing buffer. Viable leukocytes were then counted with trypan blue and added to the microculture at various concentrations.

Microculturing. Single-cell suspensions were cultured in 60-well Terasaki plates (Nunc, Roskilde, Denmark) at a total volume of 10 μl per well and at concentrations ranging from 5×10^3 to 5×10^4 leukocytes per well. In some experiments, antigenic stimulation and exogenous T cell help were provided to the microculture by the allospecific helper T cell clone D10.G4.1 (American Type Culture Collection, Rockville, Md.), which is reactive with *I-A^b^* molecules on B6C3 lymphocytes (31). However, no difference was seen in the numbers of Ag-specific cells in cultures in the presence or absence of exogenous D10.G4.1 T cells. These lung and spleen cell preparations were not depleted of endogenous T cells. The culture medium for microculturing was RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 50 mg of gentamicin sulfate, 50 U of penicillin, and 50 μg of streptomycin per liter. The Terasaki plates were placed in a humidified modular incubator chamber (ICN Flow, Costa Mesa, Calif.) within a 5% CO_2 incubator at 37°C. After 1 week in culture, the supernatants from each well were harvested and analyzed by ELISA for the presence of specific Abs to FHA.

ELISA testing. For each experiment, pools of individual serum, BAL, and NW samples from five mice were tested. Immulon I plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with Ags diluted in coating buffer (21) at the following doses: 50 μl of FHA (5 μg/ml) or LOS (2 μg/ml) per well or 100 μl of PT (4 μg/ml), pertactin (2 μg/ml), or fimbriae (2 μg/ml) per well. Pooled serum samples were added to the coated plates starting at a 1:100 dilution, pooled BAL and NW samples were added at a 1:2 dilution, and twofold dilutions were performed over 12 wells. For determination of total IgG and total IgA in BAL and NW samples, plates were coated with 50 μl of 2-μg/ml goat anti-mouse Ig (Southern Biotechnology Associates, Inc., Birmingham, Ala.) per well, and samples were added as for Ag-specific Ig titer determinations. Plates were incubated as previously described (32) with alkaline phosphatase-conjugated goat anti-mouse IgG (goat anti-mouse IgG-AP) (Southern Biotechnology Associates) or goat anti-mouse IgA-AP. The optical densities were read 30 min after the addition of Sigma 104 phosphatase substrate (Sigma Chemical Co., St. Louis, Mo.) by use of an EL312 reader (Bio-Tek Instruments, Winooski, Vt.) at a dual wavelength of 405 to 550. An endpoint titer was calculated for each sample by extrapolation to zero from the linear portion of the titration curve.

Fifty microliters of a dilution of the microculture supernatant (10 μl of supernatant diluted in 50 μl of RPMI 1640 containing 0.04% sodium azide) was added to each well of FHA-coated plates. Plates were treated as described above; goat anti-mouse Ig-AP was used for the detection of wells containing specific Abs of any Ig class to FHA, or goat anti-mouse IgM-AP, IgG-AP, or IgA-AP was used for the detection of wells containing isotype-specific Abs to FHA. A standard curve was generated by use of the IgG1 anti-FHA monoclonal Ab M08 X3C or M08 X3E, provided by Elizabeth Leininger, Division of Bacterial Products, U.S. Food and Drug Administration, Bethesda, Md. An optical density more than twice that in control wells (RPMI 1640 containing sodium azide but not culture supernatant) was considered positive.

Cell staining. Representative samples of the cell populations added to the microcultures were stained with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse Ig (Ig-FITC) (Becton Dickinson, Mountain View, Calif.) or goat anti-mouse Ig-FITC, IgM-FITC, IgG-FITC, or IgA-FITC (Southern Biotechnology Associates) and analyzed by flow cytometry (FACScan; Becton Dickinson) to determine the numbers of B cells.

Statistics. The frequency of FHA-specific B cells in each lymphocyte preparation, based on the number of microculture wells positive for the production of FHA-specific Abs, was calculated by use of Poisson distribution statistics (16, 26). For lungs and tracheas from aerosol-challenged mice, which were cultured at four dilutions, one 60-well plate per dilution, a graph of the fraction of negative wells versus the number of input cells was constructed. The point at which 37% of the wells were negative was the cell input number, at which there was one FHA-reactive cell per well. For control lungs and for both challenged and control spleens, which yielded low or negative responses at the highest dilution tested, the Poisson formula was applied directly (16, 26).

PCR. The polymerase chain reaction was performed on samples from the lungs of aerosol-challenged mice at intervals of 1 to 26 weeks after aerosol challenge. Following homogenization of the lungs in 5 ml of PBS in a stomacher (Lab-Blender 80; Tekmar Co., Cincinnati, Ohio) and treatment with dispase for 2 h, an aliquot of each of these samples was centrifuged at a low speed to remove cell debris. The supernatant was collected and centrifuged at a high speed in an Eppendorf centrifuge for 10 min. The pellet was resuspended in PCR buffer (The Perkin-Elmer Corp., Norwalk, Conn.). A pair of 24-mer PCR primers (P1 and P2) was derived from a noncoding region upstream of the open reading frame of the *B. pertussis* porin gene. P1 was synthesized from nucleotide bases 119 through 142 (5′-3′), and P2 was synthesized from nucleotide bases 254 through 277 (3′-5′) (19). PCR generates a 159-bp product with these primers (18). Two-temperature (94 and 65°C) PCR was performed by use of a DNA thermal cycler (Perkin-Elmer

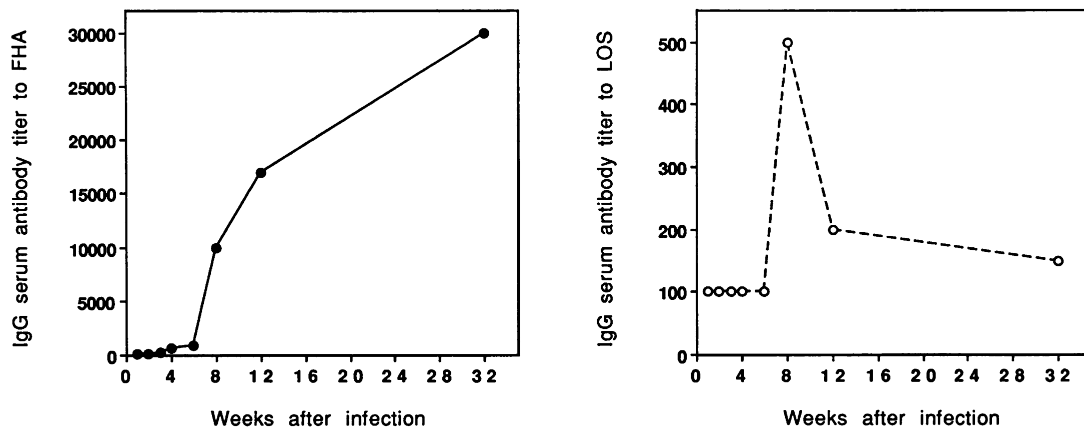

FIG. 1. IgG titers in the serum of mice aerosol challenged with *B. pertussis*. Titers were determined for pools of serum from five mice and expressed as the reciprocal of the endpoint dilution, calculated by extrapolation to zero from the linear portion of the titration curve. Titers to FHA (●) increased steadily from weeks 3 to 32; titers to LOS (○) first appeared at week 8 and then declined.

Cetus) for 35 cycles, and 30 μl of the amplified DNA product was run on a 2% agarose gel. A positive result was determined by viewing the 159-bp PCR product stained with ethidium bromide. This PCR method is specific, since no PCR product was identified on an ethidium bromide-stained agarose gel after amplification of DNA from a number of unrelated gram-negative and gram-positive bacteria. For further enhancement of the sensitivity, standard Southern blotting (22) was performed. PCR products were denatured and then transferred from the agarose gel to a nitrocellulose filter. A DNA probe was prepared by labelling the 159-bp PCR product amplified from *B. pertussis* chromosomal DNA with ^{32}P by use of the random-primer DNA-labeling system (BRL Life Technologies, Inc., Gaithersburg, Md.). DNA hybridization was performed at 60°C overnight.

Leukocytosis. For determination of total leukocyte counts in the blood of infected mice, 5 μl of blood was taken from the periorbital sinus with a capillary pipet and immediately transferred to 10 ml of Hematall isotonic diluent (Fisher Diagnostics, Pittsburgh, Pa.), and leukocyte counts were determined on a Coulter Counter ZM (Coulter Electronics, Inc., Hialeah, Fla.) after lysing of erythrocytes with Hematall LAS reagent (Fisher).

RESULTS

Abs in serum and respiratory tract secretions. Specific Abs to Ags of *B. pertussis* were measured in serum, BAL, and NW samples at 0 to 32 weeks after infection with *B. pertussis*. Most time points were tested again for serum Abs, with similar results. No Abs to PT, pertactin, or fimbriae were detected. A moderate IgG anti-LOS response appeared in the serum at week 8 and declined thereafter (Fig. 1). IgG anti-FHA Ab was first detected in serum and BAL samples at low levels at 3 weeks postinfection; the Ab levels then increased, and high levels could be detected as late as 32 and 26 weeks, respectively (Fig. 1 and Table 1). IgA anti-FHA Ab was first detected in BAL samples at 3 weeks postinfection but not in serum samples at any time point examined (Table 1). IgM anti-FHA Ab was not detected in any of the samples at any of the time points analyzed. Total IgG and IgA levels in the BAL and NW samples were measured and found to vary over time, with the total IgG levels in the BAL samples ranging from titers of 150 to 1,000 and peaking 2 weeks after aerosol challenge. Therefore, the specific titers were not normalized to the concentration of total IgG or IgA (32). Peak leukocytosis was also seen at 2 weeks after aerosol infection (66,263 leukocytes per μl of blood in aerosol-challenged mice versus 39,205 leukocytes per μl in control mice; $P < 0.05$), a result suggesting that PT released by *B. pertussis* in the lungs at this time may affect lymphocyte recirculation and capillary permeability.

Characterization of lung and spleen cell populations. To determine whether the specific Ab detected in the BAL samples was synthesized locally, we examined the ability of lymphocytes isolated from the lungs and tracheas of infected mice to produce specific Ab to FHA in vitro. Cells isolated from the spleens and from the lungs and tracheas were stained with Abs specific for B lymphocyte markers and analyzed by flow cytometry. Lungs and tracheas yielded an average of 17×10^6 leukocytes per mouse; 13 to 19% of these were B cells. Spleens yielded an average of 70×10^6 leukocytes per mouse; 35 to 37% of these were B cells. Cells from the lungs and tracheas of infected mice in six experiments were also analyzed for surface Ig (sIg) isotype. These cells were 18.3% $sIgM^+$, 16.7% $sIgG^+$, and 4.4% $sIgA^+$.

Enumeration of FHA-specific B cells. The frequency of

TABLE 1. Serum and mucosal Abs to FHA after respiratory infection with live *B. pertussis*

Wk after infection	Titer[a] of the indicated Ab in the following samples:					
	Serum		BAL		NW	
	IgG	IgA	IgG	IgA	IgG	IgA
0	<100	<100	<2	<2	<2	<2
1	<100	<100	<2	<2	<2	<2
2	<100	<100	<2	<2	<2	<2
3	200	<100	8	4	<2	<2
4	600	<100	12	6	<2	<2
6	900	<100	20	8	<2	<2
8	10,000	<100	200	50	3	<2
12	17,000	<100	300	50	3	<2
26	10,100	<100	200	100	8	3
32	30,000	<100	ND[b]	ND	ND	ND

[a] Reciprocal of the endpoint dilution, calculated by extrapolation to zero from the linear portion of the titration curve; pools of samples from five mice were used. IgM anti-FHA Ab was not detected in any of these samples. No Ab to FHA was detected in the fluids of control mice.

[b] ND, not done.

TABLE 2. Enumeration of FHA-specific B lymphocytes after respiratory infection with live *B. pertussis*

Wk after infection	No. of FHA-specific cells per 10^6 B lymphocytes from[a]:			
	Lungs and tracheas[b]		Spleens[c]	
	Infected	Uninfected	Infected	Uninfected
1	<2.1	<2.7	<1.1	<0.87
2	14	<2.3	3.4	4.5
3	190	<5.3	9.5	10.0
4	88	ND[d]	1.4	1.6
6	312	<5.2	11.3	6.4
8	127	<2.3	<0.99	0.84
12	712	<3.9	<0.88	1.1
13	963	ND	ND	ND
25	931	<8.4	2.8	7.4

[a] "Less than" values indicate that all wells were negative, even at the highest concentration of leukocytes per well.
[b] Pools of lungs and tracheas from six infected or four uninfected mice.
[c] Pools of spleens from three mice.
[d] ND, not done.

FHA-specific B cells was determined by Poisson analysis (16, 26). The numbers of FHA-specific B cells in the lungs and tracheas of infected mice increased 2 to 3 weeks postinfection and continued to increase, remaining high 13 to 25 weeks postinfection (Table 2), while the numbers of FHA-specific B cells in the lungs and tracheas of uninfected mice were consistently low. There was no increase in the frequency of FHA-specific B cells in the spleens of infected mice (Table 2). Several time points of the time course were tested again, with similar results.

Ig classes of FHA-specific B cells. For determination of the Ig classes of the FHA-specific B cells from the lungs and tracheas of aerosol-challenged mice, some microculture supernatants were developed in an ELISA with goat anti-mouse isotype-specific Ig–AP reagents (Table 3). IgG- and IgA-secreting B cells were both detected at all three time points; no IgM-secreting B cells were seen at any time point.

Characterization of the persistent FHA response. For determination of whether the persistent response was due to resting memory B cells, which require division following in vitro stimulation, lung and trachea cells were irradiated with 3,000 rads before being added to microcultures. Irradiation did not ablate the number of FHA-specific Ab-producing cells in lungs from infected mice in three experiments, in either the presence or the absence of allospecific stimulation by D10.G4.1 cells (there were 30 FHA-specific cells per 10^6 irradiated B cells versus 43 FHA-specific cells per 10^6 nonirradiated B cells at 3 weeks after infection; 550 FHA-specific cells per 10^6 irradiated B cells versus 751 FHA-specific cells per 10^6 nonirradiated B cells at 13 weeks after infection; and 328 FHA-specific cells per 10^6 irradiated B cells plus D10.G4.1 cells versus 963 FHA-specific cells per 10^6 nonirradiated B cells plus D10.G4.1 cells at 13 weeks after infection).

TABLE 3. Ig classes of FHA-specific B lymphocytes in lungs following respiratory infection with live *B. pertussis*

Wk after infection	No. of FHA-specific cells per 10^6 B lymphocytes[a]		
	IgM(μ)	IgG(γ)	IgA(α)
3	<1.94	71.89	35.43
6	<1.62	54.88	32.18
13	<3.08	280.5	328.6

[a] Ig classes of supernatants from replicate cultures developed with isotype-specific reagents; each experiment was done with a pool of cells from six mice.

TABLE 4. Detection of *B. pertussis* in lungs and tracheas by bacterial culture and PCR techniques

Wk after infection and sample	Bacterial recovery[a]		PCR[b]	
	No. infected/total	Log_{10} CFU	Ethidium bromide staining	Southern blotting
1	3/3		3/3	ND[c]
1		6.78		
2		6.75		
3		6.77		
4	4/5[d]		0/5	5/5
1		0.70		
2		4.67		
3		1.30		
4		1.04		
5		2		
8	1/16	1[e]	0/16	12/16
26	ND	ND	1/16	6/16

[a] Colony growth on Bordet-Gengou plates of individual lung samples before dispase treatment.
[b] Number of animals positive in the PCR analysis/total number of animals (lung samples after dispase treatment).
[c] ND, not done.
[d] Sample 1 was negative for *B. pertussis* and scored as 0.5 colony per plate.
[e] Log_{10} CFU from the one positive animal.

Evidence for the presence of persistent Ags in the lungs. The detection after a single infection of a persistent FHA response that was radiation resistant suggested that this response may have been due to persistent Ags. The recovery of *B. pertussis* from the lungs of mice at different times after infection is shown in Table 4. The percentage of mice with recoverable viable bacteria, as well as the total number of recoverable bacteria per mouse, decreased from weeks 1 to 4, and by week 8, only 1 of 16 mice had recoverable bacteria.

PCR analysis of lung tissue samples from mice at different times after infection showed that *B. pertussis*-specific DNA could be detected in all mice for up to 4 weeks after infection, with declining numbers of positive mice at 8 weeks, but even at 26 weeks, 6 of 16 mice (37.5%) were positive for *B. pertussis*-specific DNA (Table 4). Previous experiments with seeded cultures showed the limit of detectability to be 1 to 10 CFU with this PCR technique (17).

DISCUSSION

FHA is a high-molecular-weight filamentous protein that is one of the major adhesins of *B. pertussis* (14). Aerosol challenge of B6C3 mice with *B. pertussis* induced little specific Ab response until 2 to 3 weeks postinfection, at which time serum and mucosal Abs to FHA were detected (Table 1). An increase in total IgG levels in BAL samples, possibly due to increased capillary permeability, and peak leukocytosis were also observed 2 weeks postinfection. PT has been described to cause increases in capillary permeability and leukocytosis (25, 34). Thus, the delay in the Ab response to FHA following respiratory infection with *B. pertussis* may have been due to alterations in immunity due to the effects of PT.

Serum and mucosal Abs rose to high levels in these mice between 8 and 32 weeks postinfection (Table 1). High-titer serum IgG Ab responses to FHA (endpoint titer, 10,000) have also been observed in our laboratory for BALB/c mice as late as 12 weeks following *B. pertussis* aerosol infection (1).

Mice immunized parenterally or mucosally with highly purified FHA show high levels of serum and mucosal Abs to FHA as well as a decrease in infection following aerosol challenge (14, 32). Adult B6C3 mice immunized mucosally with highly purified FHA show a 2- to 3-log reduction in bacterial recovery from the respiratory tract, 63% of these mice clearing the infection completely from their tracheas (32).

Although no Ab response to PT was detected in the serum or secretions of B6C3 mice, high titers of anti-PT serum Abs have been detected in BALB/c mice following *B. pertussis* aerosol infection (1, 12). Thus, the absence of anti-PT Abs in B6C3 mice following infection may have been due to the mouse strain used. Differences in the ability of PT to sensitize mice to histamine challenge have been observed between C3H mice (resistant) and BALB/c and C57BL/6 mice (sensitive) (2).

High frequencies of anti-FHA Ab-secreting cells isolated from the lungs of mice were detected between 6 and 25 weeks postinfection, a result indicating that a portion of the specific Abs detected in the BAL samples following infection was due to local synthesis. Following respiratory immunization, IgG responses in the lungs have been described for several systems (7, 23, 29, 38). Local production of IgG in the lungs may reflect the transmission of Ags by lung macrophages through the lymphatics to the lymph nodes that drain the lungs (13) as well as antigenic stimulation of lymphocytes resident in the lungs. The observation that anti-FHA Ab-secreting cells were detected in the lungs but not in the spleens of infected mice may have been due to the fact that FHA is a high-molecular-weight filamentous protein that may not be easily disseminated from the respiratory tract. This suggestion would predict that the source of the observed anti-FHA serum IgG following infection was the respiratory tract (including draining lymph nodes) rather than peripheral lymphoid organs.

The observation of local synthesis of anti-FHA Abs in the respiratory tract led us to investigate whether the source of this long-lived specific Ab response in the lungs might have been due to FHA-specific memory B lymphocytes generated during the initial infection. The fact that the anti-FHA Ab response in the B cell population isolated from the lungs of infected mice was radiation resistant suggested to us that the response in culture was due to cells, including activated plasma cells and plasmablasts, already stimulated in vivo. Chronic stimulation of anti-FHA Ab responses following respiratory infection may be due to either the persistence of viable *B. pertussis* in the respiratory tract or the persistence of FHA Ags.

To examine the possibility of persistent infection, we cultured lungs for viable pertussis organisms and used the PCR technique to detect *B. pertussis*-specific DNA. The latest time point at which we were able to culture bacteria from the lungs of infected mice was 8 weeks after aerosol infection. Although *B. pertussis*-specific DNA was found by PCR in 75% of the mice at 8 weeks and 37.5% of the mice at 26 weeks after aerosol infection, it is not clear whether this result was due to quiescent bacteria that were not able to be cultured or to dead bacteria. Gray and Cheers (11) previously found only one in four mice with viable organisms 63 days after intranasal infection with *B. pertussis*. Recent studies have shown that *B. pertussis* can be internalized by various cells. In vitro studies have demonstrated that *B. pertussis* is internalized by HeLa cells (8) and, in the presence of specific Abs, by human polymorphonuclear leukocytes (35). In mice challenged intranasally with *B. pertussis*, bacteria were observed in the focal plane of alveolar macrophages in lung sections as early as 7 days after immunization (4). Macrophage-associated bacteria were still observed as late as 34 days following intranasal challenge of mice. In vivo studies showed that in BAL samples from three children who were infected with human immunodeficiency virus and who had pertussis, the bacteria were associated with pulmonary alveolar macrophages; this association appeared to be intracellular, on the basis of reduced levels of fluorescence (3). Thus, a persistent low-level infection may contribute to the observed long-lived Ab response; chronic stimulation of an immune response by *B. pertussis* cells internalized in alveolar macrophages requires further study.

A second mechanism that may account for the long-term generation of Ag-specific plasma cells and plasmablasts following aerosol infection is the persistence of FHA that has been elaborated by *B. pertussis* during infection. Long-lived Ab responses following a single exposure to Ag have been attributed to the repeated stimulation of Ag-specific B cells by persistent Ag (10). Trapping and retention of Ag on follicular dendritic cells following immune complex formation has been described as one mechanism of persistent Ab stimulation (36). FHA, a high-molecular-weight filamentous protein demonstrated to be a major adhesin of *B. pertussis*, contains a potential lectin binding site (6) in addition to an arginine-lysine-aspartic acid (RGD) sequence that binds to particular integrins, most notably CR3 (27). Therefore, the functional binding sites present on FHA may also mediate interactions with nonphagocytic Ag-presenting cells, resulting in the long-lived production of anti-FHA Abs. Studies are currently in progress to search for FHA in situ in infected tissues.

Following *B. pertussis* infection in infants, few specific anti-FHA Abs are initially detected, although anti-FHA Ab levels increase with time, reaching maxima that persist as long as 52 weeks postinfection (20). Thus, the long-lived Ab response that we described for mice following a single respiratory infection with *B. pertussis* may be a paradigm for the specific immune response in infants following pertussis infection. This model system affords the opportunity for further study of the chronic interactions of bacterial Ags with the immune system.

ACKNOWLEDGMENTS

We thank Mary Leef and Mayumi Endoh for collection of serum and pulmonary secretions and Pat Carter for many of the FACScan analyses. We are grateful to Dennis Klinman and Hilda Marcus for careful and critical review of the manuscript.

REFERENCES

1. **Amsbaugh, D. F.** Unpublished data.
2. **Bergmann, R. K., and J. Munoz.** 1968. Action of the histamine sensitizing factor from *Bordetella pertussis* on inbred and random bred strains of mice. Int. Arch. Allergy **34:**331–338.
3. **Bromberg, K., G. Tannis, and P. Steiner.** 1991. Detection of *Bordetella pertussis* associated with the alveolar macrophages of children with human immunodeficiency virus infection. Infect. Immun. **59:**4715–4719.

4. **Cheers, C., and D. F. Gray.** 1969. Macrophage behaviour during the complaisant phase of murine pertussis. Immunology **17:**875–887.
5. **Cherry, J. D., P. A. Brunell, G. S. Golden, and D. T. Karson.** 1988. Report of the task force on pertussis and pertussis immunization—1988. Pediatrics **81:**939–984.
6. **Delisse-Gathoye, A.-M., C. Locht, F. Jacob, M. Raaschou-Nielsen, I. Heron, J.-L. Ruelle, M. de Wilde, and T. Cabezon.** 1990. Cloning, partial sequence, expression, and antigenic analysis of the filamentous hemagglutinin gene of *Bordetella pertussis*. Infect. Immun. **58:**2895–2905.
7. **Eldridge, J. H., J. K. Staas, J. A. Meulbroek, J. R. McGhee, T. R. Tice, and R. M. Gilley.** 1990. Disseminated mucosal anti-toxin antibody responses induced through oral or intrathecal immunization with toxoid-containing biodegradable microspheres, p. 375–378. *In* T. T. MacDonald, S. J. Challacombe, P. W. Bland, C. R. Stokes, R. V. Heatley, and A. M. Mowat (ed.), Advances in mucosal immunology. Kluwer Academic Publishers, London.
8. **Ewanowich, C. A., A. R. Melton, A. A. Weiss, R. K. Sherburne, and M. S. Peppler.** 1989. Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. Infect. Immun. **57:**2698–2704.
9. **Farizo, K. M., S. L. Cochi, E. R. Zell, E. W. Brink, S. G. Wassilak, and P. A. Patriarca.** 1992. Epidemiological features of pertussis in the United States, 1980–1989. Clin. Infect. Dis. **14:**708–719.
10. **Gray, D., and H. Skarvall.** 1988. B-cell memory is short-lived in the absence of antigen. Nature (London) **336:**70–73.
11. **Gray, D. F., and C. Cheers.** 1969. The sequence of enhanced cellular activity and protective humoral factors in murine pertussis immunity. Immunology **17:**889–896.
12. **Halperin, S. A. (Dalhousie University, Halifax, Nova Scotia, Canada).** 1992. Personal communication.
13. **Harmsen, A. G., B. A. Muggenburg, M. B. Snipes, and D. E. Bice.** 1985. The role of macrophages in particle translocation from lungs to lymph nodes. Science **230:**1277–1280.
14. **Kimura, A., K. T. Mountzouros, D. A. Relman, S. Falkow, and J. L. Cowell.** 1990. *Bordetella pertussis* filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. Infect. Immun. **58:**7–16.
15. **Lambert, H. J.** 1965. Epidemiology of a small pertussis outbreak in Kent County, Michigan. Public Health Rep. **80:**365–369.
16. **Lefkovits, I., and H. Waldmann.** 1984. Limiting dilution analysis of the cells of the immune system. I. The clonal basis of the immune response. Immunol. Today **5:**265–268.
17. **Li, Z.-M.** Unpublished observation.
18. **Li, Z.-M., S. A. Halperin, D. L. Jansen, T. M. Finn, C. R. Manclark, and M. J. Brennan.** 1992. Identification of pertussis in children using molecular techniques, abstr. D-131, p. 117. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992.
19. **Li, Z.-M., J. H. Hannah, S. Stibitz, N. Y. Nguyen, C. R. Manclark, and M. J. Brennan.** 1991. Cloning and sequencing of the structural gene for the porin protein of *Bordetella pertussis*. Mol. Microbiol. **5:**1649–1656.
20. **Long, S. S., C. J. Welkon, and J. L. Clark.** 1990. Widespread silent transmission of pertussis in families: antibody correlates of infection and symptomatology. J. Infect. Dis. **161:**480–486.
21. **Manclark, C. R., B. D. Meade, and D. G. Burstyn.** 1986. Serological response to *Bordetella pertussis*, p. 388–394. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), Manual of clinical laboratory immunology, 3rd ed. American Society for Microbiology, Washington, D.C.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Analysis of recombinant DNA clones, p. 363–401. *In* T. Maniatis, E. F. Fritsch, and J. Sambrook (ed.), Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Mason, M. J., N. A. Gillett, and D. E. Bice.** 1989. Comparison of systemic and local immune responses after multiple pulmonary antigen exposures. Reg. Immunol. **2:**149–157.
24. **Mertsola, J., O. Ruuskanen, E. Eerola, and M. K. Viljanen.** 1983. Intrafamilial spread of pertussis. J. Pediatr. **103:**359–363.
25. **Munoz, J. J.** 1985. Biological activities of pertussigen (pertussis toxin), p. 1–18. *In* R. D. Sekura, J. Moss, and M. Vaughan (ed.), Pertussis toxin. Academic Press, Inc., New York.
26. **Quintans, J., and I. Lefkovits.** 1973. Precursor cells specific to sheep red cells in nude mice. Estimation of frequency in the microculture system. Eur. J. Immunol. **3:**392–397.
27. **Relman, D., E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and S. D. Wright.** 1990. Recognition of a bacterial adhesin by an integrin: macrophage CR3 ($\alpha_M\beta_2$, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. Cell **61:**1375–1382.
28. **Robinson, A., L. A. E. Ashworth, A. Baskerville, and L. I. Irons.** 1984. Protection against intranasal infection of mice with *Bordetella pertussis*. Dev. Biol. Stand. **61:**165–172.
29. **Rose, F. V., and J. J. Cebra.** 1985. Isotype commitment of B cells and dissemination of the primed state after mucosal stimulation with *Mycoplasma pulmonis*. Infect. Immun. **49:**428–434.
30. **Sato, Y., K. Izumiya, H. Sato, J. L. Cowell, and C. R. Manclark.** 1980. Aerosol infection of mice with *Bordetella pertussis*. Infect. Immun. **29:**261–266.
31. **Schrader, C. E., A. George, R. L. Kerlin, and J. J. Cebra.** 1990. Dendritic cells support production of IgA and other non-IgM isotypes in clonal microculture. Int. Immunol. **2:**563–570.
32. **Shahin, R. D., D. F. Amsbaugh, and M. F. Leef.** 1992. Mucosal immunization with filamentous hemagglutinin protects against *Bordetella pertussis* respiratory infection. Infect. Immun. **60:**1482–1488.
33. **Shahin, R. D., M. J. Brennan, Z. M. Li, B. D. Meade, and D. R. Manclark.** 1990. Characterization of the protective capacity and immunogenicity of the 69 kDa outer membrane protein of *Bordetella pertussis*. J. Exp. Med. **171:**63–73.
34. **Spangrude, G. J., B. A. Braaten, and R. A. Daynes.** 1984. Molecular mechanisms of lymphocyte extravasation. I. Studies of two selective inhibitors of lymphocyte recirculation. J. Immunol. **132:**354–362.
35. **Steed, L. L., M. Setareh, and R. L. Friedman.** 1991. Intracellular survival of virulent *Bordetella pertussis* in human polymorphonuclear leukocytes. J. Leukocyte Biol. **50:**321–330.
36. **Szakal, A. K., M. H. Kosco, and J. G. Tew.** 1988. A novel in vivo follicular dendritic cell-dependent iccosome-mediated mechanism for delivery of antigen to antigen-processing cells. J. Immunol. **140:**341–353.
37. **Thomas, M. G., L. A. E. Ashworth, E. Miller, and H. P. Lambert.** 1990. Nasal immunoglobulin A responses to pertussis toxin, filamentous hemagglutinin and agglutinogens 2 and 3 after infection with *Bordetella pertussis* and immunization with whole-cell pertussis vaccine, p. 330–334. *In* C. R. Manclark (ed.), Proceedings of the Sixth International Symposium on Pertussis. Department of Health and Human Services publication no. 90-1164. Department of Health and Human Services, Bethesda, Md.
38. **Weissman, D. N., D. E. Bice, D. W. Siegel, and M. R. Schuyler.** 1990. Murine lung immunity to a soluble antigen. Am. J. Respir. Cell Mol. Biol. **2:**327–333.