

Protective Immunity to *Bordetella pertussis* Requires Both B Cells and CD4⁺ T Cells for Key Functions Other than Specific Antibody Production

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Abstract

To investigate the fundamental nature of protective immunity to *Bordetella pertussis*, we studied intranasal immunization of adult mice with formalin-fixed *B. pertussis* (FFBP), followed by aerosol *B. pertussis* challenge. Mice given two doses of FFBP intranasally completely cleared a subsequent pertussis aerosol challenge from tracheae and lungs (defined as protection), but there was no correlation between levels of specific antibody and clearance of bacteria. Further, transfer of immune serum before aerosol challenge had minimal effects on bacterial burdens. However, pertussis-specific T cells producing interferon γ but not interleukin 4 or interleukin 10 were detected in draining lymph nodes of FFBP-immunized mice. Significantly, repeated immunization of B cell knockout (BKO) mice resulted in partial protection, and complete protection was reconstituted by transfer of pertussis-immune B cells; reconstituted BKO mice had little if any detectable antipertussis antibodies. Immunization of mice lacking all T cells or lacking CD4⁺ T cells did not lead to protection; in contrast, CD8⁻ mice were protected. Mice depleted of CD4⁺ T cells after immunization but before aerosol challenge, which thus had normal amounts of specific antibodies, were not optimally protected. Taken together, these data indicate that protective immunity to pertussis is dependent on both CD4⁺ T cells and B cells, and both cell types provide significant functions other than specific antibody production.

Key words: pertussis • immunization • protective immunity • B cell • T cell

Introduction

Bordetella pertussis is a gram-negative bacterium that typically infects mammals through inhalation, establishing a respiratory infection in the nasopharynx, trachea, and bronchial tree of the lungs (1). The resulting disease, whooping cough, is associated with significant morbidity and mortality in children worldwide; adult disease is generally milder, but adults may serve as reservoirs for further infection (1, 2). Although older studies suggested that protection against pertussis after natural infection was relatively long lived (3), others suggest that protection after either vaccination or natural infection may wane by young

adulthood (2, 4, 5). Vaccination with whole cell vaccines provides good protection against childhood pertussis infection and has largely controlled whooping cough in industrialized countries (3). In the United States, acellular vaccines comprised of purified pertussis antigens are now replacing whole cell vaccines.

Despite many years of vaccine use, the nature of protective immunity to pertussis induced by either natural infection or vaccination remains poorly understood. Early clinical trials of whole cell pertussis vaccines suggested that protection occurred in the presence of high titers of agglutinating antibodies, but in clinical trials of acellular vaccines it has been difficult to define quantitative correlations between specific antipertussis antibody levels and protection against disease (6–9). On the other hand, passive transfer of various types of antipertussis antibodies has been shown to protect against pertussis infection in animal models (10–14). Mice genetically deficient in mature B cells given aerosol

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B. pertussis infection develop a persistent infection that never resolves but does not disseminate (15). In addition, recent studies have demonstrated that pertussis-specific human (16) and murine (17, 18) T cells, particularly CD4⁺ T cells, secrete IL-2 and IFN- γ in response to specific stimulation. In a mouse model of respiratory infection, transfer of these Th1-like cells resulted in bacterial clearance in the apparent absence of antibodies (17). Further, after aerosol infection, mice lacking IFN- γ did not control bacterial growth well (19), and mice lacking IFN- γ receptors had disease with aberrant organ pathology, atypical dissemination of bacteria outside of lungs, and occasional deaths (15). Consistent with controversial reports suggesting that *B. pertussis* survives, if not replicates, within murine (20), rabbit (21), or human (21) macrophages, the latter results suggest that cell-mediated immunity may play a significant role in controlling pertussis infection.

To further define the fundamental basis of protective immunity to pertussis, we have used a mouse model of pertussis infection that mimics the severity of human disease. Infection of adult mice with pertussis through an aerosol chamber deposits bacteria on the ciliated epithelium of the trachea and the bronchial tree; bacteria multiply and reach peak numbers by days 7–10, and decline thereafter until clearance 40–100 d later (22). On the other hand, infection of mice younger than 19 d old is not controlled and results in death after \sim 3 wk. Although infection of neonatal mice is obviously of interest, it is difficult to obtain sufficient cells or serum for comprehensive analysis from such young mice. The long time course of primary infection clearance in adult mice limits studies of secondary immunity, and immunodeficient mice do not clear primary infection (15, 19). Here, we have used intranasal immunization of various mice with formalin-fixed *B. pertussis* (FFBP)¹ followed by aerosol pertussis challenge to comprehensively evaluate the relative contributions of T cells, B cells, and specific antibody to secondary protective immunity.

Materials and Methods

Mice. BALB/cAnNcR, BALB/c.nu/nu (nude), C3H/HeJ, and C57BL/6 mice were obtained from the Animal Production Program, Division of Cancer Research Treatment, National Cancer Institute. Total TCR knockout (KO) mice (*Tar β -Tar δ* ⁻) and CD4 KO mice, both on a B6/129 background, were purchased from the Induced Mutant Resource of The Jackson Laboratory. β 2-microglobulin⁻ and *Igh6*⁻ (μ MT; reference 23) KO mice, both on a C57B/6 background, were also purchased from The Jackson Laboratory. JhD BL/6 mice (24) were obtained from Gnotobiotec Laboratory. Mice were purchased at 5 wk of age and maintained in microisolator cages under specific pathogen-free conditions, fed autoclaved food and water ad libitum, and routinely tested for common murine pathogens. In conducting the research described in this report, the investigators adhered

¹Abbreviations used in this paper: BAL, bronchoalveolar lavage; BKO, B cell knockout; FFBP, formalin-fixed *Bordetella pertussis*; FF *E. coli*, formalin-fixed *Escherichia coli*; FHA, filamentous hemagglutinin; KO, knockout; LOS, lipooligosaccharide; PRN, pertactin; PT, pertussis toxin.

to a protocol approved by the Animal Care and Use Committee of the Center for Biologics Evaluation and Research.

Bacteria and Antigens. Formalin-fixed antigens were prepared as follows: *B. pertussis* strains 18323 and Tohama I were grown on Bordet-Gengou agar plates, and confluent lawns of bacteria were harvested and transferred to Stainer Scholte media. After 36–48 h of incubation (early stationary phase), bacteria were fixed by addition of formalin to a final concentration of 0.2% with gentle shaking at 24°C overnight. The preparation was then washed in PBS and resuspended to 440 μ g protein/ml (as determined by BCA protein assay; Pierce Chemical Co.) in PBS with 0.04% sodium azide as a preservative. *Escherichia coli* strain HB101 was grown on LB plates, transferred to LB liquid media, and treated as above.

Microspheres composed of poly(dl-lactide-co-glycolide) containing FFBP whole cells, filamentous hemagglutinin (FHA), pertussis toxoid, or pertactin (PRN) were prepared under Department of Health and Human Services Public Health Service contract no. 223-94-1237 by the Southern Research Institute. The encapsulated microspheres were characterized and had properties similar to those described previously (25).

Immunizations. Animals were lightly anesthetized with Metofane inhalant anesthesia (Pittman Moore, Inc.) and intranasally immunized by depositing the antigen in 25 μ l PBS on the nares as described previously (22). Mice were intranasally immunized with two doses given 1 mo apart of 1–30 μ g of formalin-fixed bacteria or 1 or 10 μ g microencapsulated pertussis antigens, as indicated. Immunized animals were administered a *B. pertussis* aerosol challenge 1 mo after the second immunization, unless otherwise stated.

Aerosol Challenge. *B. pertussis* 18323 at 10⁹/ml was administered to mice as an aerosol for 30 min as described previously (22). For each experiment, two mice were killed upon removal from the aerosol chamber to assess the initial number of viable *B. pertussis* cells in the lungs. 7 d after the *B. pertussis* challenge, which is the peak of infection in unimmunized mice given an aerosol pertussis challenge (26), lungs and tracheae of infected animals were aseptically removed, homogenized in PBS, and plated on Bordet-Gengou agar to determine the number of recoverable bacteria. Plates incubated with 100 μ l of undiluted homogenate that had no *B. pertussis* growth were scored as having 0.5 CFU/100 μ l; minimum level of detection of bacteria was therefore 1.4 log₁₀ CFU in the lungs and 0.7 log₁₀ CFU in the tracheae. Wilcoxon analysis (two-sample test assuming equal variance) was used to test bacterial recovery data for statistical significance.

Analysis of Antibody Response. Serum from mucosally immunized mice was collected by bleeding anesthetized animals from the brachial artery 1 mo after the last immunization. Bronchoalveolar lavage (BAL) fluid was collected by lavage of the lungs with sterile PBS as described previously (26). Serum and BAL fluid from immunized mice were analyzed for specific antibody as described previously (26) using Immulon 1 plates coated with pertussis antigens, serially diluted samples of sera or BAL fluid, and development with alkaline phosphatase-conjugated goat anti-mouse IgM, IgG, or IgA (Southern Biotechnology Associates, Inc.) and phosphatase substrate (Sigma-Aldrich). Specific antibody titers were expressed as the reciprocal of the endpoint dilution, calculated by extrapolation to zero from the linear part of the titration curve. Minimal IgM antibodies were detected at these time points, and IgG titers are shown here as indicated.

Analysis of T Cell Responses. C3H/HeJ mice were immunized intranasally with two doses given 1 mo apart of 30 μ g of formalin-fixed bacteria, as above. To visualize the lymph nodes,

mice were boosted with an additional dose of FFBP 4 d before being killed, and 24 h before being killed the mice were injected intraperitoneally with carbon soot encapsulated in lecithin (Thies Technologies). Tracheobronchial and hilar lymph nodes were removed, and T cells were enriched by passage through a nylon wool column equilibrated with 10% fetal bovine serum in RPMI 1640. Eluted T cells were collected, and enrichment was assessed by flow cytometry (see below). T cells were cultured at 2×10^5 per well in triplicate groups in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone), 2 mM glutamine, 0.075% sodium bicarbonate, and 5×10^{-5} M 2-ME (all purchased from GIBCO BRL Life Technologies) in 96-well tissue culture plates (Costar). Irradiated splenocytes (4×10^5) were added as a source of APCs, and FFBP or formalin-fixed *E. coli* (FF *E. coli*) was added as antigen at a final concentration of 50 or 10 $\mu\text{g}/\text{ml}$. For determination of proliferation, 0.5 $\mu\text{Ci}/\text{well}$ [^3H]thymidine (New England Nuclear; 6.0 Ci/mmol specific activity) was added 2 d after initiation of cultures and 16 h before harvesting and determination of incorporated radioactivity. Results are expressed as mean cpm \pm SD of triplicate cultures. For detection of cytokines, similar cultures were established in 24-well plates, using 50 $\mu\text{g}/\text{ml}$ FFBP or FF *E. coli* per well. Culture supernatants were harvested at 48 h after initiation of cultures and were tested for the presence of IFN- γ , IL-4, and IL-10 using known standards and pairs of cytokine-specific mAbs in an ELISA (BD PharMingen). In these studies, cells from immunized C3H/HeJ mice (which are also protected against aerosol challenge) were used to minimize background proliferation due to LPS recognition (particularly because FF *E. coli* was used as an irrelevant control).

Passive Transfer of Serum. Immune serum for passive transfer was prepared by immunizing large groups of mice with two doses of antigen intranasally 1 mo apart. Animals were bled at 4 wk after the second immunization, and the sera were pooled and stored at -70°C until use. Serum (2 ml) was injected intraperitoneally into normal BALB/c mice 2 h before administration of a pertussis aerosol challenge, as above. Lungs and tracheae were plated out on days 7 and 8 after the challenge.

Adoptive Transfer of Immune B Cells. Similar to T cell preparation (above), B cells were prepared from the draining lymph nodes of lungs from mice immunized with two doses of formalin-fixed bacteria given 1 mo apart, boosted 4 d before killing, and injected intraperitoneally with carbon soot. Tracheobronchial and hilar lymph nodes were removed, and B cells were enriched by treatment with anti-mouse CD8a, anti-CD4, anti-CD90, and anti-TCR- γ/δ mAbs at a concentration of 10 $\mu\text{g}/\text{ml}$ for 30 min. After washing once with DMEM, cells were resuspended in 10% rabbit complement, incubated for 30 min at 37°C , and washed three times with 10 vol DMEM before transfer. Purity of the cell population was determined using flow cytometry analysis (see below), which routinely demonstrated that enriched populations were $>90\%$ B cells, with $<3\%$ contamination with T cells. 1 mo after the second immunization, immunized recipient μMT or JhD BL/6 mice were given $2.5\text{--}4.5 \times 10^7$ B cells per animal intravenously in the lateral tail vein, boosted intranasally with formalin-fixed bacteria 1 d after transfer of B cells, and then challenged 2 wk later with an aerosol pertussis infection.

Depletion of Mice with Anti-CD4 and Anti-CD8 Antibodies and Flow Cytometry. To deplete CD4 $^+$ T cells, CD8 $^+$ T cells, or both in vivo, immunized mice were treated intraperitoneally with 1 mg of anti-CD4 mAb (clone GK1.5; rat IgG $_{2b}$), 1 mg of an anti-CD8 mAb (clone 2.43; rat IgG $_{2b}$), or 500 μg of each on days -5 and -1 relative to aerosol pertussis challenge, as well as

on day 3 after challenge. Both mAbs were precipitated from ascites with 50% ammonium sulfate. Lymph node and spleen populations from randomly chosen mice were analyzed for effectiveness of depletion on the day of the pertussis challenge and at day 7 by flow cytometry using a FACScanTM (Becton Dickinson). Cells were stained using a panel of mAbs including FITC-anti-B220, PE-anti-CD4, PE-anti-CD8, PE-anti-CD11b, and PE-anti-TCR- γ/δ (all purchased from BD PharMingen; optimal staining concentrations determined separately) in both one- and two-color staining protocols. Flow cytometry analysis of lymphocyte and spleen cells from the GK1.5- or 2.43-treated mice routinely demonstrated $>95\%$ depletion of T lymphocytes.

Results

Characterization of Protection Induced by Immunization with FFBP, and Relationship to Specific Antibody Production and T Cell Responses. To evaluate the protective capacity of FFBP, adult BALB/c mice were immunized with two intranasal doses given 1 mo apart of 1 or 10 μg FFBP in microspheres or 1 or 10 μg of unencapsulated FFBP and were challenged by exposure to a *B. pertussis* aerosol 4 wk after the last immunization. Animals given 1 or 10 μg microencapsulated FFBP exhibited a dramatic 4 \log_{10} CFU reduction in bacterial recoveries from their lungs as well as a 3 \log_{10} CFU reduction in bacterial recoveries from their tracheae for both 1 and 10 μg doses compared with the bacterial recoveries from the lungs of unimmunized infected control mice (Table I). Mice administered two doses of 10 μg of unencapsulated FFBP intranasally completely cleared the pertussis infection from both the lungs and tracheae in this experiment. Protection by FFBP was dose dependent: mice immunized with 1 μg of FFBP were not as well protected as those immunized with 10 μg . Thus, subsequent experiments used 10–30 μg doses of unencapsulated FFBP given intranasally. For comparison, a 30- μg dose of FFBP is equivalent to $\sim 3 \times 10^8$ bacteria.

To evaluate the specificity of protection, the protective capacity of other formalin-fixed bacteria was evaluated. The strain used here for the aerosol challenge, *B. pertussis* 18323, may vary slightly in several respects from the *B. pertussis* type strain, Tohama I (27). BALB/c mice immunized intranasally with 10 μg of FFBP derived from the strain Tohama I and then challenged with *B. pertussis* strain 18323 had a similar reduction in bacterial recoveries from the lungs and tracheae compared with animals immunized and challenged with *B. pertussis* strain 18323, indicating that the FFBP Tohama I strain provided comparable protection against the 18323 challenge strain. However, mice immunized with 10 μg FF *E. coli* and challenged with *B. pertussis* 18323 had no significant decrease in infection (Table I).

To examine the relationship between protection and antibody levels, serum samples were obtained from FFBP-immunized mice just before challenge and were analyzed for the presence of various antipertussis antibodies (Table II). The amount of circulating antibody to pertussis antigens at the time of the pertussis challenge was compared with the subsequent bacterial recoveries on day 7 after challenge in

Table I. Bacterial Recoveries after *B. pertussis* Aerosol Challenge of Mice Immunized Intranasally with FFBP or FF *E. coli*

Group	Lungs		Tracheae	
	Log ₁₀ CFU	No. infected/total	Log ₁₀ CFU	No. infected/total
10 µg <i>B. pertussis</i> microspheres	2.25 ± 1.29*	2/6	1.23 ± 0.76*	2/6
1 µg <i>B. pertussis</i> microspheres	2.30 ± 0.73*	5/6	1.33 ± 0.43*	5/6
10 µg FFBP 18323	1.40 ± 0.00*	0/6	0.70 ± 0.00*	0/5
1 µg FFBP 18323	4.52 ± 0.26*	6/6	1.75 ± 0.33*	5/5
10 µg FFBP Tohama I	1.74 ± 0.55*	6/6	0.91 ± 0.43*	6/6
10 µg FF <i>E. coli</i>	5.90 ± 0.35	6/6	4.22 ± 0.50	6/6
Infected controls	6.42 ± 0.27	6/6	4.88 ± 0.22	6/6

Mice were immunized twice intranasally as indicated and challenged with *B. pertussis*. Values shown are the mean ± SD log₁₀ CFU 1 wk after aerosol challenge. Results from one representative experiment of three experiments of similar design are shown.

**P* < 0.005 compared with unimmunized infected controls.

individual mice. All of the immunized mice had no detectable bacteria in lungs or tracheae at 7 d after challenge. In contrast to our previous studies using microencapsulated purified pertussis antigens, which elicited high levels of antigen-specific IgG and IgA in serum and secretions (25), two intranasal immunizations with 30 µg FFBP did not induce consistent levels of specific serum IgG or IgA to purified pertussis antigens FHA, PRN, pertussis toxin (PT), lipooligosaccharide (LOS), or fimbriae (Table II). In studies of >30 individual animals, occasional small IgG serum antibody responses to all individual antigens were observed (data not shown). However, all animals immunized produced an excellent serum IgG antibody response to pertussis whole cell lysate, although no measurable levels (<1/50)

of specific serum IgA to pertussis antigens were observed. Analysis of BAL samples of similarly immunized mice showed that few animals produced antibody responses in the lung to the purified antigens, but when these lung lavage samples were reacted with whole cell lysate, all animals had strong specific IgG (1:120 ± 45) and IgA (1/450 ± 60) titers.

The ability of various antibody preparations, including one shown previously to transfer protection (25), to protect mice against a *B. pertussis* infection by passive immunization was then evaluated. As shown in Table III, mice that received the serum antibody to the microsphere combination completely cleared the infection in both the lungs and tracheae by 7 d after the challenge. In contrast, animals that

Table II. Relationship between Serum Antibody Response and Bacterial Clearance in FFBP-immunized Mice

Animal no.	Bacterial recovery		Serum IgG antibodies to:*					Whole cell lysate
	Lungs	Tracheae	FHA	PRN	PT	LOS	Fimbriae	
	<i>log₁₀ CFU</i>							
1-1	Sterile	Sterile	–	–	–	–	–	19,000
1-2	Sterile	Sterile	–	1,000	–	–	–	10,000
1-3	Sterile	Sterile	–	4,000	–	–	–	40,000
1-4	Sterile	Sterile	16,000	1,000	–	–	–	70,000
1-5	Sterile	Sterile	2,500	1,000	–	–	–	40,000
1-6	Sterile	Sterile	1,500	–	–	–	–	90,000
1-7	Sterile	Sterile	800	–	–	–	–	40,000
1-8	Sterile	Sterile	–	800	–	–	–	40,000
Controls	6.49	4.94	–	–	–	–	–	–

Mice were immunized twice intranasally with FFBP and bled 1 mo later, and all mice were then challenged with *B. pertussis*. Values shown are the mean ± SD log₁₀ CFU 1 wk after aerosol challenge, or were below the limit of detection and designated as sterile. Results from 8 individual mice of >30 individual mice assessed are shown.

*Values shown are endpoint titers. A dash indicates that no specific antibody was detected (titer of <1:50); no antigen-specific serum IgA was detected in any sample.

Table III. Bacterial Recoveries after Passive Transfer of Serum

Serum from mice immunized with:*	n	log ₁₀ CFU [‡]	
		Lungs	Tracheae
Microsphere combination	5	1.40 ± 0.00	0.70 ± 0.00
30 μg FFBP	6	5.65 ± 0.36	3.62 ± 0.20
30 μg FF <i>E. coli</i>	5	6.26 ± 0.24	4.38 ± 0.16
Normal mouse serum	6	6.69 ± 0.15	4.88 ± 0.14
Infected controls	7	6.32 ± 0.46	4.73 ± 0.30

*Mice received 2 ml of serum intraperitoneally as indicated 2 h before individual serum samples were obtained, then were challenged with *B. pertussis*. Endpoint serum IgG titers of the pooled sera injected intraperitoneally (2 ml) were as follows. For the anti-microsphere combination serum: anti-FHA and anti-PT, 70,000 each, and anti-PRN, 45,000; for the anti-30 μg FFBP serum: anti-FFBP, 40,000, and anti-FHA, 2,400. After transfer and immediately before challenge, the geometric mean serum titers in recipient mice were as follows. For the anti-microsphere combination serum: anti-FHA, 41,000, anti-PT, 36,000, and anti-PRN, 37,000; for the anti-FFBP serum: anti-FFBP, 36,000, and anti-FHA, 1,100. Results from one representative experiment of two experiments of similar design are shown.

[‡]Values shown are the mean ± SD log₁₀ CFU 1 wk after aerosol challenge.

received 2 ml of serum from mice immunized with 30 μg of FFBP did not have a significant reduction in colonization in the lungs, compared with levels of bacteria in infected control mice or mice given normal mouse serum. This immune serum contained high amounts of antibodies to pertussis whole cell lysates, as well as minimal levels of antibodies to FHA (Table III footnote).

To determine whether antigen-specific T cell responses were present in FFBP-immunized mice, purified T cells were prepared from mice immunized intranasally with FFBP and cultured with a source of APCs as well as with FFBP as antigen. FFBP-stimulated T cells proliferated vigorously (Fig. 1); no proliferation above background was observed in cultures stimulated with an irrelevant antigen (*FF E. coli*) or in cultures lacking APCs. Further, T cells from unimmunized mice did not proliferate in response to either antigen (Fig. 1). To examine the cytokines produced by such pertussis-specific T cells, supernatants were harvested from comparable parallel cultures. By 48 h, supernatants from cultures containing pertussis-immune T cells, FFBP, and APCs contained 2.2 ± 0.3 ng/ml IFN-γ, but levels of IL-4 and IL-10 in the same supernatants were below the limit of detection (50 pg/ml and 30 pg/ml, respectively). All three cytokines were undetectable in all other control cultures (50 pg/ml for IFN-γ).

Defective Generation of Protection in B Cell KO Mice, and Reconstitution of Protection by Transfer of Primed B Cells. We further assessed the requirement for B cell-dependent protection after immunization with FFBP or microspheres by immunizing μMT B cell KO mice (BKO [23]) and matched wild-type control C57BL/6 mice intranasally with

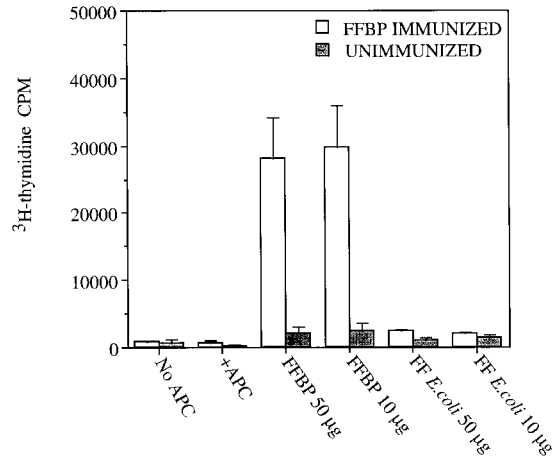


Figure 1. Antigen-specific proliferation of T cells in FFBP-immunized mice. Cells obtained from the draining lymph nodes of FFBP-immunized mice were cultured in the presence or absence of APCs, FFBP at the indicated doses, or *FF E. coli* at the indicated doses. Proliferation was assessed by overnight pulse of [³H]thymidine added at day 2 after initiation of cultures and harvest on day 3. Mean cpm ± SD of triplicate cultures, from FFBP-immunized (white bars) or unimmunized (gray bars) are shown. T cells used in this experiment were 97% CD4⁺CD8⁺ T cells and 0.9% B220⁺ cells.

FFBP. *B. pertussis* aerosol challenge was administered 1 mo after the second immunization. Both groups of wild-type control mice, immunized with either microencapsulated pertussis antigens or with FFBP, had a substantial reduction in infection in the lungs and complete clearance of infection from the tracheae (Fig. 2). These immunocompetent

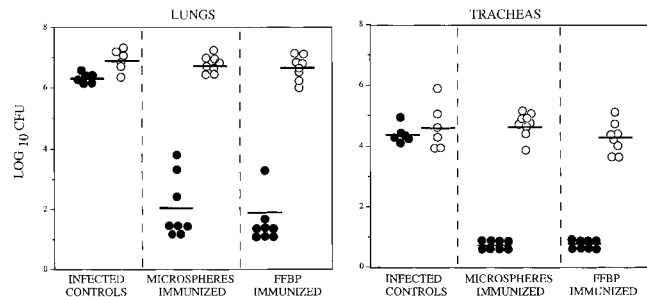


Figure 2. Protection after immunization with FFBP in B cell KO mice. Wild-type C57BL/6 mice (●) and C57.*Igh6*^{-/-} B cell KO mice (○) were immunized twice with either pertussis antigens in microspheres or with FFBP, and were challenged 1 mo later along with unimmunized wild-type or BKO control mice (infected controls). Bacterial recoveries as log₁₀ CFU from lungs and tracheae 1 wk after pertussis infection are shown. Circles represent results from individual mice; bars represent median values. Results from one representative experiment of three experiments of similar design are shown. By Wilcoxon analysis, median recoveries of bacteria in both lungs and tracheae are significantly different between wild-type mice and BKO mice immunized with microspheres ($P < 0.05$) or with FFBP ($P < 0.05$). Median bacterial recoveries are also significantly different between infected control wild-type mice and wild-type mice vaccinated with either microspheres ($P < 0.05$) or FFBP ($P < 0.05$). Median bacterial recoveries were not significantly different between infected BKO control mice and immunized BKO mice ($P > 0.05$), or between infected wild-type control mice and infected BKO control mice in either lungs or tracheae ($P > 0.05$).

FFBP-immunized animals had average serum IgG titers against whole cell lysate of 47,500. Immunocompetent mice immunized with the microsphere combination had high serum titers against all three pertussis antigens (FHA, 50,000; PT, 100,000; PRN, 50,000). By comparison, BKO mice immunized with either microencapsulated antigens or with FFBP had no reduction in bacterial recoveries compared with the unimmunized infected control mice, and had no detectable levels of serum antibody to pertussis antigens (<1:50).

To assess the relative contribution of B cells versus specific antipertussis antibodies to clearance of pertussis infection, reconstitution experiments were performed. Because large quantities of anti-FFBP antibody had little impact on bacterial recoveries even in intact wild-type mice (Table III), only transfer of B cells was studied in BKO mice. Two types of B cell-deficient mice, both JhD mice (experiment 1; reference 24) derived by targeted deletion of the Jh region of the IgM heavy chain, and μ MT mice (experiments 2 and 3; reference 23) derived by targeted deletion of the μ region, were studied in these experiments. B cells were prepared from draining lymph nodes of mice immunized with two doses of FFBP intranasally, and were transferred to recipient mice that were also immunized twice with FFBP intranasally before transfer. To stimulate engraftment, all mice were given a third immunization with FFBP intranasally 1 d after B cell transfer, then were challenged 2 wk later. Of particular note, although BKO mice immunized with FFBP had no reduction in colonization after a pertussis challenge (Fig. 2), similar BKO mice developed a significant ability to control an infection if immunized three times with FFBP (2 log₁₀ CFU reduction; Fig. 3).

This was despite having no detectable specific antibody at the time of challenge, as expected (Table IV). Adoptive transfer of primed B cells obtained from the draining lymph nodes of vaccinated immunocompetent C57B/6 mice then enabled similarly immunized animals to completely clear the infection in two of three experiments performed (Fig. 3). BKO mice immunized with FFBP were completely protected if given between $3\text{--}4.5 \times 10^7$ FFBP primed B cells (experiments 1 and 2), but not if given only 2.5×10^7 primed B cells (experiment 3). In experiments (1 and 2) in which reconstitution was effective, serum antibody titers to pertussis whole cell lysate at the time of challenge were evaluated in individual mice. Very low titers were observed in all cases except for groups of immunized wild-type control mice, and there was no correlation between production of serum antipertussis antibodies and clearance of bacteria (Table IV). Notably, 7/12 (58%) of the KO mice that received primed B cells in the two positive experiments had very low but detectable serum antibody levels, but 5/12 (42%) had undetectable levels of specific antibodies and cleared infection (Table IV). Specific serum antibody titers did not increase after challenge (footnote to Table IV).

Defective Generation of Protection in T Cell KO Mice. To assess the requirement for T cells in the mechanism of immune clearance after a mucosal immunization, both adult BALB/c athymic *nu/nu* mice and mice lacking all TCRs (*Ta β -Ta δ* ⁻) were studied. B6129/J.*Ta β -Ta δ* ⁻ mice that lack all mature T cells and control B6129/J F₂ mice were given two immunizations intranasally with 30 μ g of FFBP and an aerosol *B. pertussis* challenge 1 mo later, and bacterial recoveries in lungs were evaluated. As shown in Fig. 4

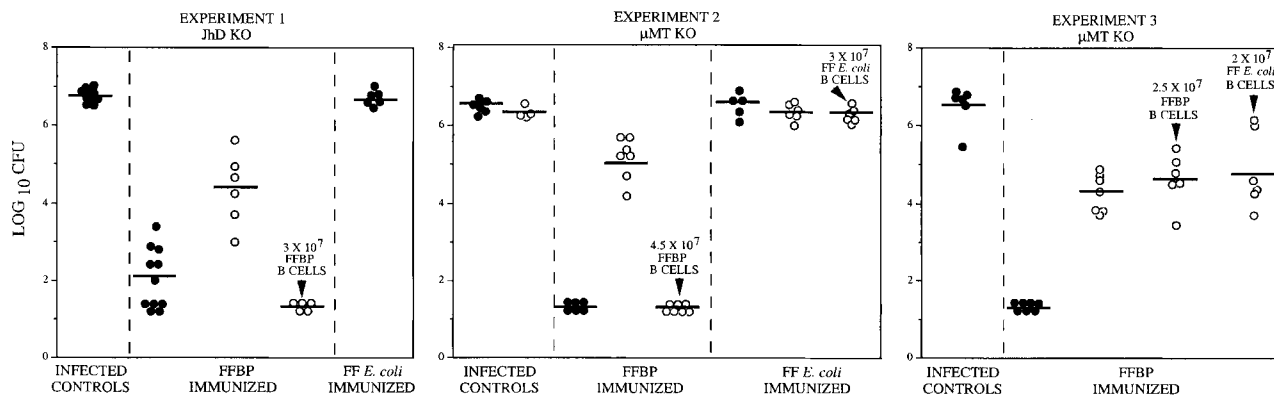


Figure 3. Reconstitution of protection after immunization with FFBP in B cell KO mice by transfer of FFBP-primed B cells. Wild-type C57BL/6 mice (●) and JhD or μ MT BKO mice (BKO mice indicated by ○) were immunized twice with FFBP. B cells were prepared from similar mice immunized twice with FFBP or FF *E. coli* as indicated, and were transferred to immunized BKO mice that were then given a third immunization with FFBP and an aerosol challenge with *B. pertussis*. Bacterial recoveries as log₁₀ CFU from lungs 1 wk after pertussis infection for all three individual experiments are shown; similar results were seen in bacterial recoveries from tracheae (data not shown). Circles represent results from individual mice; bars represent median values. Results of all three individual experiments are shown. By Wilcoxon analysis, median recoveries of bacteria in lungs are significantly different between wild-type mice and BKO mice immunized three times with FFBP ($P < 0.05$; Wilcoxon analysis), as are median bacterial recoveries between reconstituted and nonreconstituted BKO mice in experiments 1 and 2 ($P < 0.05$). Median bacterial recoveries are also significantly different between infected control wild-type mice and wild-type mice vaccinated with FFBP in all three experiments ($P < 0.05$). In experiment 2, median bacterial recoveries were not significantly different between infected wild-type control mice or infected BKO control mice in either lungs or tracheae ($P > 0.05$). In experiment 2, median bacterial recoveries were not significantly different between infected control mice and either FF *E. coli*-immunized mice or BKO mice that were reconstituted with FF *E. coli*-primed B cells ($P > 0.05$).

Table IV. Anti-*B. pertussis* Antibody Titers in Reconstituted BKO Mice

Group	Experiment 1*	No. responding/total	Experiment 2*	No. responding/total
C57BL/6 immunized FFBP	58,000 ± 15,000	11/11	50,000 ± 11,000	6/6
C57BL/6 immunized FF <i>E. coli</i>	<50	0/6	<50	0/5
C57BL/6 infected controls	<50	0/10	<50	0/6
BKO immunized FFBP	<50	0/6	<50	0/7
BKO immunized FFBP;				
FFBP-primed B cells transferred	266 [‡]	3/5	675 [‡]	4/7
BKO immunized FF <i>E. coli</i>	ND	-	<50	0/6
BKO immunized FF <i>E. coli</i> ;				
FF <i>E. coli</i> -primed B cells transferred	ND	-	<50	0/6
BKO infected controls	ND	-	<50	0/4

*Mice were treated as described in the legend to Fig. 3, and individual serum samples were obtained immediately before aerosol pertussis challenge. Geometric mean titers ± SD of IgG serum antibodies tested by ELISA on plates coated with FFBP are shown for the groups of mice in Fig. 3. For numbers of responding mice, responses are designated as positive for titers of 1:50 or greater. Data from experiment 3 is not shown, as reconstitution with this lower number of B cells did not result in bacterial clearance. All animals had serum IgG titers of <50 for FHA, PT, and PRN, except for the C57BL/6 immunized FFBP group in experiment 2. 2/6 mice had anti-PRN IgG titers of 6,000 and 11,000.

[‡]Individual serum IgG titers for experiment 1 immediately before aerosol pertussis challenge were 200, 300, <50, 300, and <50. Individual titers for experiment 2 were <50, <50, 300, 200, 1,500, <50, and 700. Individual serum IgG titers 1 wk after challenge (for the same mice, listed in the same order) for experiment 1 were 500, 300, <50, 200, and <50. Individual titers for experiment 2 were <50, <50, 600, <50, 600, 400, and <50.

A, control C57BL/6 animals were protected against a pertussis challenge; however, the immunized TCR KO mice were not protected at all. Similar results were observed in bacterial recoveries in the tracheae (data not shown).

Similarly, athymic BALB/*c.nu/nu* mice lacking TCR- α/β^+ T cells and control euthymic BALB/*c* mice were immunized with 30 μ g of FFBP and challenged. Unlike immunized normal mice, immunized BALB/*c.nu/nu* mice

were unable to clear the infection at 1 wk and had bacterial recoveries similar to the unimmunized BALB/*c.nu/nu* and unimmunized BALB/*c* mice (Fig. 4 B). Similar results were observed in the tracheae (data not shown).

The long-term outcome of immunization of BALB/*c.nu/nu* mice with FFBP was studied further as well. Here, immunized mice were challenged and assessed for bacterial burdens at 3 and 5 wk (rather than 1 wk) after challenge.

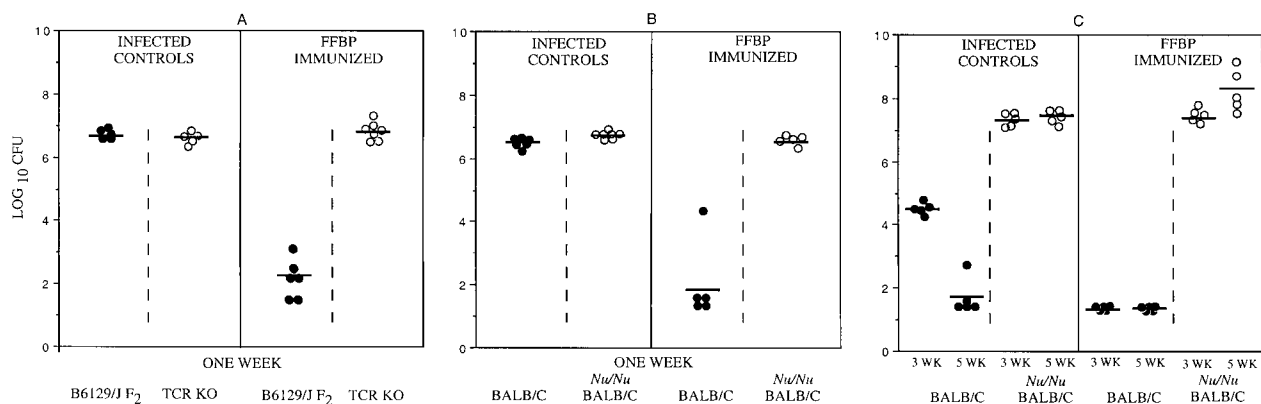


Figure 4. Protection after immunization with FFBP in T cell-deficient mice. Wild-type B6/129 F2 or BALB/*c* mice, as indicated (●) and B6/129 *Tαβ⁻Tαδ⁻* mice or BALB/*c.nu/nu* mice, as indicated (○) were immunized twice with FFBP, then challenged with *B. pertussis*. Bacterial recoveries as log₁₀ CFU from lungs 1 wk (A and B) or 3 and 5 wk (C) after pertussis infection are shown. Circles represent results from individual mice; bars represent median values. Results of all three individual experiments are shown. By Wilcoxon analysis, median recoveries of bacteria in lungs are significantly different between wild-type mice and T cell KO or *nu/nu* mice vaccinated with FFBP ($P < 0.05$). Median bacterial recoveries are also significantly different between infected control wild-type mice and wild-type mice immunized with FFBP ($P < 0.05$). Median bacterial recoveries were not significantly different between either type of infected control mice and immunized T cell KO mice (A; $P > 0.05$), nor between infected control mice and FFBP-immunized *nu/nu* mice (B; $P > 0.05$). Median bacterial recoveries were significantly different between wild-type BALB/*c* mice and *nu/nu* mice, both infected and immunized, at both time points (C; $P < 0.05$).

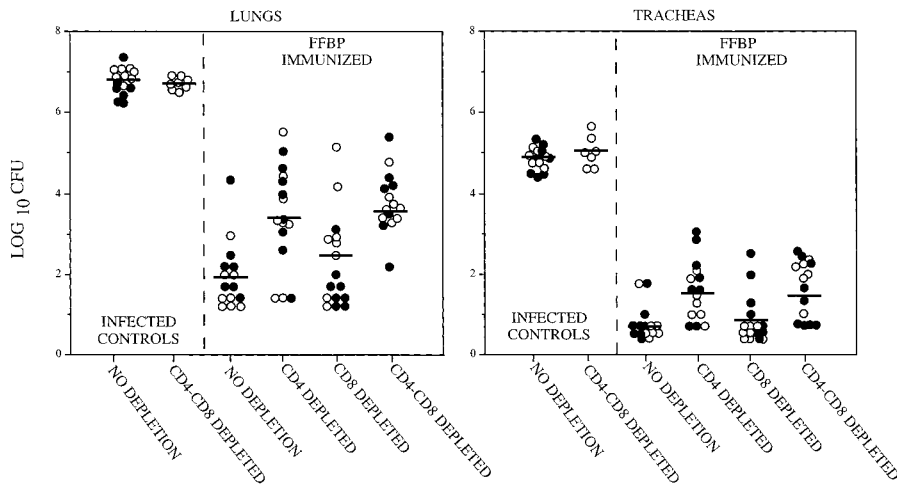


Figure 5. Protection after immunization with FFBP in mice depleted in vivo of CD4⁺ T cells, CD8⁺ T cells, or both. BALB/c mice were immunized twice with either antigens in microspheres or with FFBP. 5 d before aerosol pertussis challenge, in vivo depletion of T cell subsets was initiated, as indicated. Bacterial recoveries as log₁₀ CFU from lungs and tracheae 1 wk after pertussis infection are shown. Results from both experiments, of similar design, are shown; circles represent results from individual mice (●, experiment 1; ○, experiment 2), and bars represent median values. By Wilcoxon analysis, median recoveries of bacteria in both lungs and tracheae are significantly different between nondepleted mice and either CD4-depleted mice or CD4/CD8-depleted mice immunized with FFBP for both lungs and tracheae ($P < 0.05$). Median bacterial recoveries are also

significantly different between infected control mice and nondepleted mice immunized with FFBP ($P < 0.05$). Median bacterial recoveries are also significantly different between infected control (either nondepleted or CD4/CD8-depleted) mice and CD4-depleted mice immunized with FFBP ($P < 0.05$). Median bacterial recoveries were not significantly different between nondepleted immunized mice and CD8-depleted immunized mice ($P > 0.05$), nor between infected wild-type control mice and infected CD4/CD8-depleted control mice in either lungs or tracheae ($P > 0.05$).

At these later time points, the pertussis infection not only persisted but increased in both unimmunized BALB/c.nu/nu and immunized BALB/c.nu/nu mice (Fig. 4 C). In contrast, all immunized BALB/c mice had cleared the infection completely at 3 wk, and even unimmunized infected control BALB/c mice had almost completely resolved the infection at week 5. Groups of eight animals, both immunized and unimmunized, were also challenged and observed to determine survival. As observed previously (17), both groups of BALB/c.nu/nu mice began dying at 5 wk. By 11 wk after the aerosol challenge, only one of eight of each of these two groups survived, in contrast to BALB/c immunized and control mice, which all survived. At that time, lungs from the two surviving BALB/c.nu/nu mice were assessed for bacterial burden, and had bacterial recoveries of 7.3 and 7.5 log₁₀ CFU, respectively.

To assess the contribution of T cell subpopulations to the expression of protection, BALB/c mice were immunized intranasally with 30 μg of FFBP and depleted of CD4⁺ cells, CD8⁺ cells, or both and then challenged. Spleens and tracheobronchial lymph nodes from treated mice analyzed by flow cytometry for depletion of T cells at the time of challenge contained <2% residual CD4⁺ and/or CD8⁺ T cells, as appropriate. Control unimmunized BALB/c mice that were challenged or depleted of both CD4⁺ and CD8⁺ T cells before challenge each averaged 6.6 log₁₀ CFU in their lungs. In contrast, immunized and challenged intact mice, as well as CD8-depleted mice, exhibited a 4 log or greater average reduction in colonization (Fig. 5). Bacterial burdens in both lungs and tracheae of mice immunized and then depleted of CD4⁺ cells, or total T cells, were higher than in immunized mice but lower

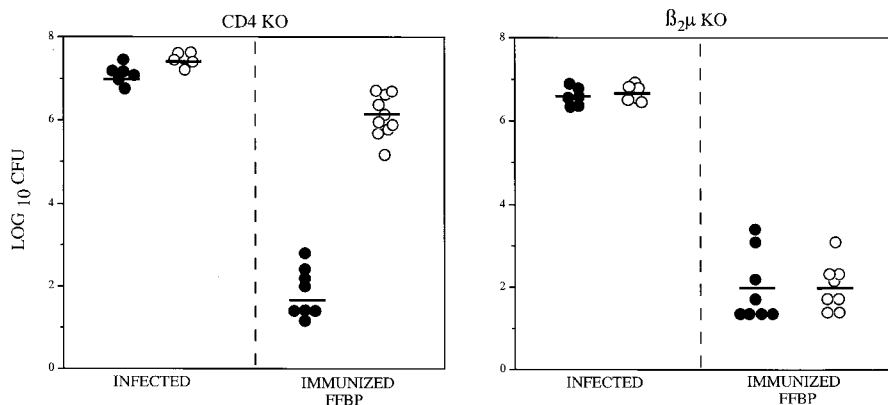


Figure 6. Protection after immunization with FFBP in mice genetically lacking CD4⁺ T cells or CD8⁺ T cells. B6/129 F2 mice (background strain for CD4 KO mice; A), C57BL/6 mice (background strain for β2μ⁻ KO; B), CD4 KO mice, and β2μ⁻ KO mice (CD8-deficient mice) were immunized twice with FFBP and challenged with *B. pertussis*. Bacterial recoveries as log₁₀ CFU from lungs 1 wk after pertussis infection are shown. Results from one representative experiment of two experiments of similar design are shown. ● indicates results from individual wild-type mice, and ○ indicates results from individual KO mice; bars represent median values. By Wilcoxon analysis, median recoveries of bacteria in both lungs and tracheae are significantly different between infected control mice and wild-type mice immunized with FFBP (A and B; $P < 0.05$), and between infected control CD4 KO mice and immunized CD4 KO mice (A). Median bacterial recoveries are not significantly different between wild-type mice and β2μ⁻ KO (CD8-deficient) immunized mice ($P > 0.05$), or between infected wild-type control mice or the respective infected KO control mice (A and B; $P > 0.05$).

different between wild-type B6/129 F2 mice and CD4 KO mice immunized with FFBP (A; $P < 0.05$). Median bacterial recoveries are also significantly different between infected control mice and wild-type mice immunized with FFBP (A and B; $P < 0.05$), and between infected control CD4 KO mice and immunized CD4 KO mice (A). Median bacterial recoveries are not significantly different between wild-type mice and β2μ⁻ KO (CD8-deficient) immunized mice ($P > 0.05$), or between infected wild-type control mice or the respective infected KO control mice (A and B; $P > 0.05$).

than in unimmunized mice; thus, CD4-depleted mice did not clear the infection effectively.

Further experiments were performed using CD4 KO or β 2-microglobulin KO mice, which lack mature CD8⁺ cells. CD4 KO mice and appropriate control B6/129 F₁ mice were immunized twice intranasally with FFBP and challenged. Fig. 6 shows that immunized control animals averaged a 5 log₁₀ CFU reduction in bacterial recoveries compared with the unimmunized infected controls or with unimmunized infected CD4 KO mice. However, the immunized CD4 KO mice had only a 1 log₁₀ CFU average decrease in bacterial recovery from the lungs. In contrast, when β 2-microglobulin KO mice and control C57BL/6 mice were similarly immunized, the KO mice were as well protected as the control mice (Fig. 6).

Discussion

Understanding of the nature of protective immunity to *B. pertussis* is of interest not only for knowledge of basic mechanisms, but also in designing vaccine strategies. Here, we show that similar to natural infection, protection induced by intranasal immunization with FFBP was dose dependent and specific for pertussis, but not limited to one pertussis strain (Table I). Although specific antipertussis antibodies were detected using whole bacteria as antigen, minimal amounts of antibodies specific for major pertussis antigens were detected (Table II), and transfer of large amounts of anti-FFBP antibodies had minimal impact on bacterial burdens after aerosol pertussis challenge, even in normal wild-type mice (Table III). Antigen-specific, pertussis-responsive T cells that proliferated and produced IFN- γ but not IL-4 or IL-10 were readily detected in the draining lymph nodes after intranasal immunization with FFBP (Fig. 1; Results text), which are most likely CD4⁺ Th1-like T cells. However, intranasal immunization of BKO mice with FFBP did not lead to development of protective immunity, as assessed by clearance of bacteria, after aerosol challenge (Fig. 2). Surprisingly, protection in BKO mice was readily reconstituted by transfer of pertussis-immune B cells shortly before challenge (Fig. 3). Similarly, immunization of mice lacking all T cells (Fig. 4) or lacking CD4⁺ T cells (Figs. 5 and 6) did not lead to protection against aerosol challenge; in contrast, CD8-deficient mice (Figs. 5 and 6) were protected. Thus, the results presented here indicate that pertussis-specific B cells and CD4⁺ T cells, but not CD8⁺ T cells, are critical to the optimal generation and expression of secondary protective immunity to pertussis after aerosol immunization with whole formalin-fixed bacteria.

The relative contributions of CD4⁺ and CD8⁺ T cells were examined using two methods: study of immunized and challenged normal mice that were depleted of T cell subpopulations, and study of immunized and challenged KO mice. Both approaches lead to the same conclusion. Neither CD8-depleted mice nor β 2-microglobulin KO mice, which lack mature CD8⁺ T cells, differed from intact mice in their ability to control aerosol pertussis challenge

after immunization with FFBP (Figs. 5 and 6). In fact, the CD8 depletion experiments serve as an internal control demonstrating that the depletion manipulations did not in and of themselves alter the outcome of infection after immunization and challenge. In contrast, immunized CD4 KO mice were completely unable to control infection, whereas depletion of CD4⁺ T cells after immunization but before challenge compromised the degree of protection but did not ablate it entirely. Immunized wild-type mice depleted of CD4⁺ T cells just before challenge had specific antibodies just as normal control mice, and thus the presence of specific antibodies at the time of challenge was apparently insufficient for optimal protection in CD4-depleted mice.

These results are consistent with other studies also indicating crucial roles for T cells in general and CD4⁺ T cells in particular. To our knowledge, this is the first report of the consequences of pertussis immunization and aerosol challenge of CD4 or CD8 KO mice, and we are not aware of studies comprehensively investigating the course of primary aerosol pertussis infection in CD4 or CD8 KO mice. Previous studies clearly indicate that athymic *nu/nu* mice, which lack all α/β ⁺ T cells, exhibit chronic infection after primary aerosol pertussis infection and die beginning at \sim 5 wk after infection (references 17, 19; Fig. 4). Similarly, *scid* mice, which lack all T cells as well as B cells, succumb to chronic pertussis infection beginning at \sim 3 wk after primary aerosol infection (19). Chronic infection in *nu/nu* mice was reversed by transfer of immune T cells (17), although the subpopulation involved in reconstitution of *nu/nu* mice was not determined. In a different experimental design, transfer of immune, pertussis-specific CD4⁺ T cells cleared primary infection in sublethally irradiated normal BALB/c mice in the apparent absence of specific antibody production, but bacterial clearance was not affected by transfer of immune, pertussis-specific CD8⁺ T cells (17). Similar to results presented here using T cells from intranasally immunized mice (Fig. 1; Results text), CD4⁺ immune T cells from mice given either a primary aerosol infection (17, 18, 28) or immunized with fixed pertussis whole cell preparations (18) also secreted IFN- γ and IL-2 in vitro in response to pertussis. Other studies clearly indicate that IFN- γ is critical to the appropriate control of bacterial dissemination, pathology, and bacterial clearance after aerosol pertussis infection of adult mice (15, 19). Further, peripheral blood mononuclear cells from pertussis-infected or convalescent children produced IFN- γ but not IL-5 in vitro in response to stimulation by heat-killed pertussis bacteria, and thus appear to have a Th1-like phenotype (16). Taken together, both previous data as well as the data presented here strongly indicate that CD4⁺ T cells contribute to protective immunity against pertussis through elaboration of Th1-like cytokines and expression of cell-mediated immunity, not just through functioning as helper cells in promoting specific antipertussis antibody production.

Mice immunized with FFBP intranasally completely cleared a subsequent pertussis aerosol challenge from tracheae and lungs, but there was no correlation between lev-

els of antibody to purified pertussis antigens (including those used in acellular pertussis vaccines) such as FHA, PRN, PT, LOS, or fimbriae and clearance of bacteria (Tables I and II). As seen previously in mice given subcutaneous (29) or intranasal (30) whole cell pertussis vaccines, immunized mice did have pertussis-specific antibodies in both serum and lung lavage samples as assessed using whole bacteria in the ELISA (Table II; Results text) that exceed levels found in naturally infected mice (17, 18, 28). Nonetheless, passive transfer of a large (2 ml) volume of immune serum obtained after immunization intranasally with FFBP had minimal effects on bacterial burdens even in normal recipient mice after aerosol challenge. This is despite the fact that serum antibody titers against FFBP in recipient mice were similar to those of intact mice at the time of challenge (Table III footnote). Similarly, in previous studies, transfer of serum from convalescent pertussis-infected mice to normal adult mice followed by aerosol pertussis infection had a minimal impact on the uptake or early growth of pertussis in lungs, and no effect on the time course of clearance (17). Further, protection against aerosol pertussis challenge was demonstrated here in the apparent absence of pertussis-specific antibodies, in that reconstituted BKO mice exhibited maximal protection but had either minimal or no detectable levels of serum antibodies (Fig. 3; Results text); similarly, mice that received immune CD4⁺ T cells had no detectable serum antipertussis antibodies, but cleared aerosol challenge (17). Also of note are studies indicating that mice given primary aerosol pertussis infection exhibited T cell-proliferative responses and bacterial clearance well before specific antipertussis antibodies were detected in serum (17, 28).

Obviously, such results are in contrast to numerous studies demonstrating that passive transfer of pertussis-specific antibodies, especially when produced by immunization with purified pertussis antigen or acellular vaccines, are able to protect against pertussis infection (10–14). We cannot exclude the possibility that undetectable pertussis-specific antibodies, produced locally after transfer of primed B cells and/or accelerated with help from primed CD4⁺ T cells upon challenge, are responsible for the observed reconstitution of protection (Fig. 3; Table IV), but we believe this is very unlikely to be the primary explanation for several reasons, including the relatively small challenge dose as well as the rapid time course of clearance. Further, BKO mice given three doses exhibited intermediate protection (Fig. 3), a situation in which no antibodies could be made. Other results suggested that BKO mice vaccinated intraperitoneally with either whole cell pertussis vaccine or acellular pertussis vaccines have diminished T cell activity (31). However, our results suggest that T cell function in these intranasally immunized BKO mice was not irreversibly compromised, or at least was still sufficient for effectiveness; importantly, reconstitution readily resulted in rapid clearance of bacteria (Fig. 3). Under these circumstances, production of specific antibodies was minimal, not increased by challenge, and indeed undetectable in the serum of 40% of successfully reconstituted BKO mice (Fig. 3; Table IV; Results text). Finally, we cannot envision a relationship be-

tween the recently described defect in the development of intestinal M cells in BKO mice (32) and the results seen here, especially because reconstitution restored clearance within 7 d after challenge. As has been proposed for the intracellular bacterium *Francisella tularensis* live vaccine strain (33), we interpret these experiments as indicating that immunity to pertussis, as generated by resolution of natural infection or by intranasal immunization with whole bacteria, involves a contribution from B cells in significant non-antibody-related roles. Although specific antipertussis antibodies may certainly contribute to protection when available, specific antibodies alone were not sufficient for optimal protection (e.g., Fig. 5); conversely, optimal protection is readily demonstrated in the apparent absence of specific antibodies (e.g., Fig. 3). This in turn may explain the difficulty in establishing definitive quantitative serological correlates of protection in pertussis vaccine trials (6–9).

The specific effector activities provided by B cells await future study, and there are several obvious candidates. These include a role in antigen presentation to immune T cells, B cell production of cytokines, B cell interaction with other cells such as macrophages or natural killer cells, and B cell regulation of appropriate cell trafficking (e.g., through production of chemokines). Obviously, not all of these possibilities are mutually exclusive, and in fact it is likely that several different effector mechanisms, including antibody production, are involved in the dependence of protective immunity to pertussis on mature B cells. The apparent requirement for pertussis-primed B cells, at least with the numbers of cells transferred here, suggests that an antigen-specific activity such as optimization of antigen presentation may be the most likely function provided by B cells. On the other hand, other studies of recirculation patterns of murine lymphocyte populations after intravenous transfer have found that very few transferred cells reach lung tissue (34–36), raising the interesting possibility that the B cell-dependent activity is operative at a distance from the site of infection and involves production of soluble mediators such as chemokines and cytokines.

These studies also again raise the controversial concept of intracellular localization of pertussis in macrophages or other cells (20, 21, 37), and may be consistent with the notion that part of the infection is extracellular and part involves intracellular infection of cells such as alveolar macrophages. Further investigation into this particular possibility appears to be well justified. Nonetheless, taken together, our studies strongly indicate that specific secondary protective immunity to pertussis is mediated by a combination of specific antipertussis antibodies, B cell function (other than antibody production), and CD4⁺ T cell-mediated immunity, including production of Th1-like cytokines. Stimulation of all these components may be the appropriate goal of vaccine development, and not all components may be invoked by vaccination with current acellular pertussis vaccines.

We thank Drs. Theresa Finn, Drusilla Burns, Suzanne Epstein, and Bruce Meade for helpful discussions and thoughtful, critical reviews

of the manuscript. This paper is dedicated to the memory of our friend and colleague, Dr. Roberta Shahin, who initiated these studies and was responsible for their intellectual direction. Her insight, knowledge, and great spirit were sorely missed in their completion.

Submitted: 23 November 1999

Revised: 7 March 2000

Accepted: 13 March 2000

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