

Cell-Mediated Immunity to *Bordetella pertussis*: Role of Th1 Cells in Bacterial Clearance in a Murine Respiratory Infection Model

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A murine respiratory infection model was used to study the mechanism of protective immunity to *Bordetella pertussis*. We found that nude mice, which are deficient in T cells, developed a persistent infection and failed to clear the bacteria after aerosol inoculation. In contrast, normal adult nonimmune mice cleared a respiratory infection approximately 35 days after challenge. Before bacterial clearance, antipertussis antibody levels in serum were low or undetectable, whereas consistent antigen-specific T-cell responses were demonstrated throughout the course of infection. The *in vitro* responses detected in immune spleen cells were mediated by a population of CD4⁺ major histocompatibility complex class II-restricted Th1-like cells that secreted interleukin-2 and gamma interferon but not interleukin-4. Adoptive transfer of immune spleen cells into nude or sublethally irradiated immunosuppressed mice before challenge resulted in bacterial clearance within 14 to 21 days. In contrast, injection of serum from convalescent mice before challenge only marginally reduced the bacterial load early in the course of infection. Furthermore, transfer of enriched T cells or purified CD4⁺ T cells but not CD8⁺ T cells from immune mice conferred a high level of protection. Recipients of CD4⁺ T cells cleared the bacteria from the lungs within 20 days of challenge, at which time *B. pertussis*-specific antibodies in the serum were undetectable. Although we do not rule out a contribution of mucosal immunoglobulin A, our findings suggest that cellular responses mediated by CD4⁺ Th1 cells play an important role in protective immunity to *B. pertussis*.

Bordetella pertussis is a gram-negative bacterium that causes whooping cough, a respiratory disease responsible for high levels of morbidity and mortality in children worldwide. Although the organism colonizes by attachment to ciliated respiratory epithelial cells and is considered to be noninvasive (3, 41, 61), a number of recent reports have suggested that it can be taken up by and survive within mammalian cells, including macrophages (11, 16, 28, 48).

After recovery from a nonlethal pertussis infection, immunity that provides long-lasting protection against subsequent disease develops (29). Immunization with the whole-cell vaccine also confers a high level of immunity and has been successful in controlling the disease in most developed countries (20, 58). However, the reactogenicity of this vaccine has motivated the search for a safer and more effective acellular pertussis vaccine (30, 43). In a recent clinical trial in Sweden, an acellular vaccine comprising chemically detoxified pertussis toxin (PT) and filamentous hemagglutinin (FHA) was found to be capable of reducing the incidence of disease, but it was less effective than whole-cell vaccines (2). Furthermore, the results of this trial failed to demonstrate a correlation between serum antibody responses to FHA or PT and protection.

Passive immunization experiments in mice have demonstrated that antibodies to several pertussis components can induce various degrees of protection against intracerebral or respiratory challenge (25, 38, 43, 46, 47). Active immunization of mice with whole-cell and acellular pertussis vaccines has been shown to induce pertussis-specific serum antibodies and protection (10, 25, 38, 43, 47). However, in a

minority of studies, such as those in which mice were immunized with the PT B oligomer (53), pertactin (52), or an attenuated *B. pertussis aroA* mutant (42), the antibody levels in serum were low or undetectable at the time of challenge but increased rapidly thereafter. Although these results are consistent with a protective role for circulating antibody transfusing into the lung after immunization or as a result of an amnestic antibody response soon after challenge, they do not rule out a contribution for local or cellular immune responses. A study that failed to find a correlation between serum antibodies and bacterial clearance after immunization of rabbits with FHA, outer membrane protein, or a whole-cell vaccine suggested that protection correlated with levels of pertussis-specific immunoglobulin A (IgA) in the lungs (4).

Indirect evidence for the role of T cells in immunity to pertussis is provided from reports that *B. pertussis*-specific T cells have been demonstrated in humans rendered immune after immunization or infection (15, 39, 40). Furthermore, a number of cases of pertussis have been documented in patients with AIDS, a disease characterized by a depletion of CD4⁺ T cells (1, 8). *B. pertussis* organisms were found inside pulmonary alveolar macrophages from these patients (8), and it has been suggested that intracellular survival of *B. pertussis* is a mechanism for persistence within the respiratory tract (8, 28, 48).

In studies of immunity to a variety of other infectious diseases, CD8⁺ and CD4⁺ T cells and subpopulations thereof have been shown to play a critical role (31). The characteristics of the disease, particularly the site of pathogen replication, determines the relative contributions of distinct T-cell subsets. Cloned murine CD4⁺ T cells have been divided into two populations on the basis of function and cytokine release (7, 34, 35). The Th1 inflammatory CD4⁺ T cells release interleukin-2 (IL-2), gamma interferon (IFN- γ), and lymphotoxin, which stimulate recruitment and

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phagocytic function of macrophages and neutrophils (26, 35, 50, 56). In addition, Th1 cells and CD8⁺ T cells are capable of killing cells infected with viruses, protozoan parasites, or intracellular bacteria such as listeriae and mycobacteria (23, 24, 31, 37, 59). Th2 helper CD4⁺ T cells secrete IL-4, IL-5, and IL-6 and are considered to be the predominant population responsible for providing the helper function for antibody responses to foreign antigens (7, 13, 35). Consequently, CD4⁺ Th1 and CD8⁺ T cells are thought to be involved in protection against intracellular pathogens through direct cell-mediated immunity, whereas CD4⁺ Th2 cells mediate protection against extracellular pathogens through the stimulation of humoral immunity (7, 35).

In this study we used a murine respiratory infection model to examine the role of T cells in immunity to pertussis. Although this is not an ideal model, the pattern of infection induced by aerosol challenge has several of the characteristics of that seen in infected children and is considered by many to be the most practicable and relevant animal model for studies of pathogenesis and immunity to pertussis (41, 45, 55). We reported previously that respiratory infection of adult mice generates strong proliferative T-cell responses against a number of pertussis antigens, including FHA, PT, a 69-kDa outer membrane protein (pertactin), and agglutinogens 2 and 3 (32). In this report we demonstrate that *B. pertussis*-specific T cells induced by infection in mice are largely confined to the CD4⁺ T-cell subpopulation that secretes IL-2 and IFN- γ and that these cells play a critical role in the clearance of bacteria after a primary infection and in the development of protective immunity against subsequent challenge.

MATERIALS AND METHODS

Mice. BALB/c (*H-2^d*), CBA (*H-2^k*), and NIH (*H-2^d*) mice were bred and maintained under specific-pathogen-free conditions. NIH and congenic B10.AQR (K^qI-A^kI-E^kD^d) mice were obtained from Olac Laboratories. Congenitally athymic nude (BALB/c *nu/nu H-2^d*) mice were obtained from Olac Laboratories and maintained in microisolators. All mice were 8 to 12 weeks old at the initiation of experiments.

Aerosol infection. Respiratory infection of mice was induced by aerosol challenge by a modification of the technique of Sato et al. (45). *B. pertussis* Wellcome 28 was grown at 36°C in Stainer-Scholte liquid medium (54). Bacteria from a 48-h culture were resuspended at a concentration of approximately 2×10^{10} CFU/ml in physiological saline containing 1% casein. The challenge inoculum was administered to mice over a period of 12 min with a nebulizer in a sealed container within a class 3 exhaust-protected cabinet. Four mice were sacrificed 2 h after aerosol challenge to assess the numbers of viable *B. pertussis* cells in the lungs after inhalation.

Enumeration of viable bacteria in the lungs. Lungs were removed aseptically and homogenized in 1 ml of sterile physiological saline with 1% casein on ice. Drops (20 μ l) of serially diluted homogenate from individual lungs were spotted in triplicate onto each of three Bordet-Gengou agar plates, and the number of CFU was estimated after 5 days of incubation. Results are mean viable *B. pertussis* counts for individual lungs from three or four mice. Unless otherwise stated, the lowest level of detection was approximately 10 CFU per lung.

Bacterial antigens. A formaldehyde-treated sonic extract of *B. pertussis* Wellcome 28 was prepared as follows. The cultured bacteria (5×10^7 to 5×10^9 /ml) were sonicated in

phosphate-buffered saline (PBS), the debris was removed by centrifugation, and the protein concentration was adjusted to 50 to 100 μ g/ml. After gelatin (0.02%, vol/vol) and Tween 20 (0.05%, vol/vol) were added, the extract was treated with 0.2 to 0.4% formaldehyde in PBS over 7 days and then dialyzed exhaustively against PBS. Heat-killed *B. pertussis* for T-cell assays was prepared by incubation of cells at 80°C for 30 min. Purified native PT, FHA, and pertactin, prepared from *B. pertussis* Tohama, were kindly provided by Carine Capiat at SmithKline Beecham, Rixensart, Belgium (9, 44).

MABs and immunofluorescence analysis. Anti-CD4 monoclonal antibody (MAB) (GK1.5) was purchased from the American Type Culture Collection. Anti-CD8 MAB (YTS 169.4) was purchased from Sera Labs Ltd. (Sussex, United Kingdom). Anti-Thy-1.2 MAB was provided by D. B. Thomas, National Institute for Medical Research, Mill Hill, London, United Kingdom. Fluorescein isothiocyanate-conjugated anti-Thy-1.2 (5a-8) was purchased from Coulter Electronics Ltd. (Luton, United Kingdom). Fluorescein isothiocyanate-conjugated goat anti-mouse Ig was purchased from Nordic Immunological Labs. Fluorescence analysis was performed by direct staining with fluorescein isothiocyanate-coupled anti-Thy-1.2 or anti-mouse Ig or by indirect staining with the rat MAB to CD4 and CD8 and then a fluorescein isothiocyanate-coupled anti-rat IgG2b MAB (NORIG) purchased from Sera Labs. Flow cytometry was performed on a Becton Dickinson FACScan.

Preparation of spleen cell subpopulations. Spleens were removed, and a single-cell suspension was prepared in RPMI 1640 medium supplemented with 10% fetal calf serum (R10 medium). Dead cells, erythrocytes, and granulocytes were removed by centrifugation at $400 \times g$ for 20 min at 20°C on an 18% metrizamide gradient prepared by diluting a 35% metrizamide (Nycomed) stock solution in H₂O with R10 medium. The viable mononuclear cells at the interface of the gradient were removed and washed twice in R10 medium. A macrophage- and B-cell-depleted (T-cell-enriched) cell population was prepared from the mononuclear spleen cells by panning on anti-mouse Ig-coated plates prepared by overnight incubation of 90-mm petri dishes with 5 ml of a solution containing 25 μ g of affinity-purified sheep anti-mouse Ig (polyvalent; Nordic Laboratories) per ml in PBS at 4°C. After four washes with PBS and two washes with R10 medium, 5 ml of the cell suspension (5×10^6 /ml) was added. The cells were incubated at room temperature for 70 min, and then the nonadherent cells were separated from the dishes and washed gently with 5-ml aliquots of R10 medium. The cells were centrifuged and resuspended at 5×10^6 /ml, and the procedure was repeated on fresh Ig-coated plates except that the plates were incubated at 37°C. The depletion technique generated a cell population consisting of approximately 80% T cells and 2 to 5% contaminating B cells. T-cell-enriched fractions were also prepared by passage through a nylon-wool column followed by panning of the nonadherent cells on Ig-coated plates. This method generated a population that was 98% T cells with less than 1% B cells.

Thy-1⁺ cells were depleted from mononuclear spleen cell preparations by incubation at 10^7 /ml for 30 min at 4°C with 1/1,000 dilution of anti-Thy-1 ascites fluid. After the cells were washed once in serum-free medium, they were resuspended in guinea pig serum diluted 1/10 in PBS as a source of complement and incubated at 37°C for 30 min. The cells were washed twice, resuspended in fresh medium, and counted. Purified CD4⁺ and CD8⁺ T cells were prepared by complement depletion of the reciprocal CD8⁺ and CD4⁺ T-cell

subpopulations from purified T cells (10^7 /ml) with anti-CD8 MAb (YTS 169.4, purified antibody, 50 μ g/ml) and anti-CD4 MAb (GK1.5, neat tissue culture supernatant), respectively. The purity of the separated fractions, determined by FACScan analysis, was 75 to 80% with less than 2% contamination with the reciprocal T-cell subset and less than 5% B cells.

Generation of pertussis-specific T-cell lines. CD4⁺ T-cell lines specific for FHA were established as previously described for influenza virus-specific T-cell clones (33). Briefly, spleen cells (2×10^6) from convalescent mice were stimulated in vitro with purified FHA (0.5 μ g/ml) for 4 days, and then fresh medium and feeder cells (2×10^6 /ml; irradiated syngeneic spleen cells) were added. After 5 to 7 more days, the surviving T cells were restimulated at 1×10^5 /ml with FHA (0.5 μ g/ml) and antigen-presenting cells (APC) (2×10^6 /ml; irradiated syngeneic spleen cells) and maintained on a 9- to 11-day cycle of antigen stimulation and rest.

Adoptive transfer of cells. Convalescent mice that had cleared the infection after aerosol challenge 6 weeks earlier were used as donors for immune spleen cells. One spleen equivalent of unseparated spleen cells from control or immune mice or the enriched B-cell or T-cell subpopulation was injected intravenously into each syngeneic recipient unless otherwise indicated. Recipients were nude mice or mice that had been irradiated 24 h earlier with a sublethal dose of 600 rads of total body irradiation from a ¹³⁷Cs source. Recipient mice were aerosol challenged 2 h after transfer. Three or four recipient mice from each experimental group of 12 to 16 animals were sacrificed at intervals between 0 and 28 days postinfection, and the viable bacteria in the lungs were enumerated.

Passive transfer of serum. Immune serum was prepared from convalescent mice that also served as the donors of immune spleen cells. Serum (0.35 to 0.45 ml) from immune or normal nonimmune (control) mice was injected intravenously into nonirradiated mice 4 to 6 h before challenge.

T-cell proliferation and lymphokine assays. Spleen cells from aerosol-infected mice were tested for in vitro proliferation against heat-killed *B. pertussis* cells (10^6 /ml), formaldehyde-treated pertussis sonic extract (5 μ g/ml), FHA (1 μ g/ml), pertactin (5 μ g/ml), and PT (0.2 μ g/ml) as previously described (32). Results are given as mean counts per minute of [³H]thymidine incorporation for triplicate cultures for groups of four to six mice.

IL-2 was assayed by testing the ability of supernatants from antigen-stimulated spleen cells (removed 24 h after the initiation of culture) to support the proliferation of the IL-2- or IL-4-dependent CTLL-2 cell line in the presence of the anti-IL-4 antibody 11B11 (10 μ g/ml). The results are expressed as counts per minute or international units (IU) per milliliter after reference to a standard curve of recombinant IL-2.

IL-4 was assayed by enzyme-linked immunosorbent assay (ELISA) with the anti IL-4 antibody 11B11 used for capture and a biotinylated polyclonal rabbit anti IL-4 antibody (PharMigen, San Diego, Calif.) for detection essentially as described by Schumaker et al. for IL-5 (49). Recombinant murine IL-4 (Genzyme) was used for the generation of the standard curve. IFN- γ was kindly assayed by A. Meager (National Institute for Biological Standards and Control), using an immunoradiometric assay as described previously (57). Levels of IFN- γ are expressed as IU per milliliter after reference to the international standard.

Phenotype and MHC restriction of the in vitro T-cell response. Purified T cells or CD4⁺ and CD8⁺ T-cell subpopulations

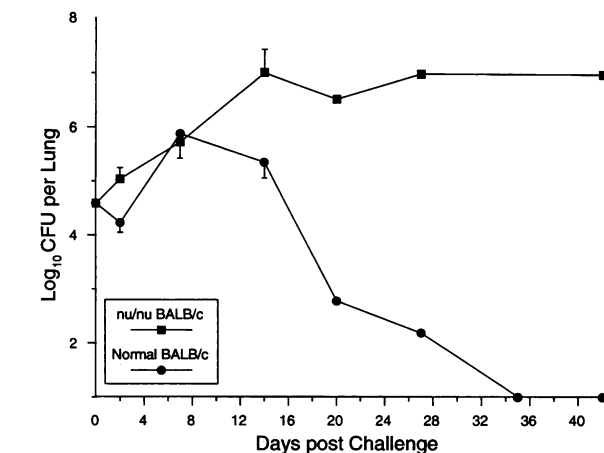


FIG. 1. Course of *B. pertussis* respiratory infection in normal and nude BALB/c mice. Euthymic or athymic (*nu/nu*) BALB/c mice were infected by aerosol challenge. Results are mean (\pm standard error) CFU in the lungs estimated for three or four mice at each time point.

were cultured at 2×10^5 /ml with antigens and syngeneic irradiated (5,000 rads) spleen cells as a source of APC. Supernatants were assayed for IL-2 24 h after the initiation of culture. Major histocompatibility complex (MHC) restriction was studied by cultivating purified T cells from CBA, BALB/c, or B10.AQR mice with antigen and irradiated APC from autologous, allogenic, or congenic donors and assessing proliferation 4 days later by determining [³H]thymidine incorporation (33).

Analysis of serum and lung Ig. The levels of antibodies to *B. pertussis* components in sera, lung lavage (22), and lung homogenates were estimated by the ELISA. Pertussis sonic extract (5 μ g/ml), PT, pertactin, and FHA (2 μ g/ml) were used to coat the plates. A serum pool derived from mice 42 days after immunization with the 3rd British Reference Preparation for whole-cell pertussis vaccine (1 IU on days 0 and 28) was used as a reference. Serum and lung samples were centrifuged and diluted in PBS-0.05% Tween 20 before the assay. Bound antibodies were detected by using alkaline phosphatase-conjugated anti-mouse IgG, IgM, and IgA (Sigma Chemical Co., Poole, Dorset, United Kingdom). The levels in serum of antibody to each *B. pertussis* antigen preparation were estimated by assigning a nominal potency of 100 U to the reference serum pool. The antibody levels, expressed in units per milliliter of undiluted serum, were determined from the reference by using a parallel-line assay. IgA antibody levels in lungs were expressed as endpoint titers after dilution of original samples prepared in 1 ml of PBS.

RESULTS

Nude mice fail to clear a *B. pertussis* respiratory infection. Delivery of *B. pertussis* Wellcome 28 to 2- to 3-month-old normal BALB/c mice by exposure to a bacterial aerosol for 12 min resulted in a highly reproducible and uniform infection. The numbers of viable bacteria recovered from the lungs of groups of mice at intervals after aerosol challenge are shown in Fig. 1. During the first few days of infection, the replication of the organism resulted in an increase in the recoverable bacteria by up to 100-fold by days 4 to 7. However, after approximately 7 days the viable counts in the

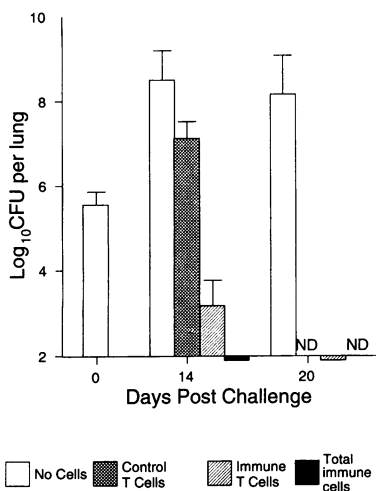


FIG. 2. Adoptive transfer of protection against pertussis in nude mice with immune T cells. Groups of nude mice were injected intravenously with one spleen equivalent of unseparated or T-cell-enriched spleen cells from normal or immune convalescent mice. A control group received no cells. Recipient mice were aerosol challenged 2 to 4 h after cell transfer, and the numbers of CFU in lungs were estimated 1 h later (day 0) and 14 or 20 days later. Results are mean (\pm standard error) CFU for individual lungs for four mice in each group at each time point. The lower level of detection at day 14 in this experiment was 100 CFU per lung. ND, not determined.

lungs slowly declined, and bacteria were undetectable 35 days after challenge.

To study the possible contribution of T cells in the pulmonary clearance of a primary pertussis infection, we also examined the course of infection in T-cell-deficient nude (BALB/c *nu/nu*) mice. In contrast to the euthymic BALB/c mice, athymic BALB/c mice developed a persistent infection and failed to clear the bacteria (Fig. 1). As in normal mice,

the levels reached a peak on day 7, but the levels remained high for at least 42 days (the longest period of study).

Adoptive transfer of protective immunity into nude mice with *B. pertussis*-immune T cells. The mechanism of protective immunity to pertussis generated by natural infection was studied by adoptive transfer of spleen cell subpopulations from immune animals. Spleen cells derived from convalescent mice that had cleared primary respiratory infection after aerosol exposure 6 weeks earlier provided a source of immune cells and will be referred to as such hereafter. Transfer of total immune BALB/c spleen cells into BALB/c athymic mice conferred on the nude mice the ability to reduce the detectable bacteria to fewer than 100 CFU per lung within 14 days after challenge (Fig. 2). At this time the viable counts in the lungs of nude mice that had received no cells or nonimmune T cells were greater than $10^{8.5}$ and 10^7 , respectively. Transfer of purified immune T cells (B-cell- and macrophage-depleted lymphocytes) also conferred a high level of protection, with a reduction in the bacterial counts to very low levels by day 14 and complete clearance 20 days after challenge. The slightly earlier clearance with the total immune spleen cells, compared with that with purified T cells, suggested that primed B cells or activated macrophages also contribute to the predominantly T-cell-mediated clearance.

Role of cellular versus humoral responses in protective immunity. Sublethally irradiated mice provided a more readily available alternative source of recipients for further adoptive transfer experiments designed to dissect the subpopulation of immune cells involved in protective immunity to *B. pertussis*. A sublethal dose of 600 rads partially depletes the immune system of mice, rendering the T cells incapable of responding to mitogens and foreign antigens, and renders the recipients more receptive to the transfer of donor cells (data not shown).

Injection of nonimmune control cells into sublethally irradiated mice followed by aerosol challenge resulted in a course of infection similar to or more protracted than that

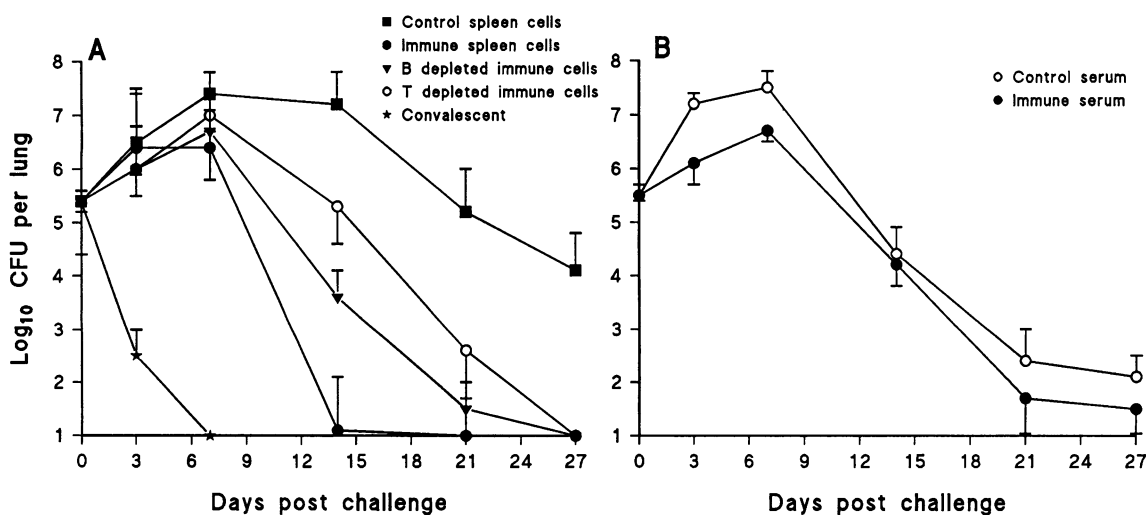


FIG. 3. Relative contributions of cellular and humoral immunity in the transfer of resistance to pertussis. (A) Groups of sublethally irradiated recipient mice ($600 \text{ rads day}^{-1}$) were injected with one spleen equivalent of unseparated spleen cells from naive control or immune convalescent mice or B-cell- or T-cell-depleted spleen cells from immune mice. A nonirradiated group of convalescent mice that were rechallenged was also included. (B) Groups of nonirradiated mice received normal serum or serum from convalescent mice. Recipients were aerosol infected 4 to 6 h after injection, and the numbers of CFU were estimated at intervals after infection. Results are mean (\pm standard error) values for individual lungs of four mice in each group at each time point and are representative of experiments repeated three times.

seen in normal naive mice (Fig. 1 and 3A). In contrast, adoptive transfer of *B. pertussis*-immune spleen cells into sublethally irradiated mice conferred on the recipients the ability to clear the bacteria within 14 to 21 days of challenge; two of four mice tested at day 14 and four of four mice tested at day 21 had no detectable bacteria in their lungs. Depletion of T cells from immune spleen cells reduced their protective capacity; recipients of T-cell-depleted immune spleen cells still had significant levels of bacteria in their lungs 14 and 21 days after challenge (Fig. 3A). However, removal of B cells also reduced the rate of clearance (Fig. 3A). These findings confirm the protective role of immune T cells demonstrated in the nude mice (Fig. 2) but also suggest that immune B cells contribute to protection, possibly through an amnestic antibody response after challenge.

Transfer of immune cells did not result in the same level of protection as that effected by the intact immune system; rechallenge of convalescent mice resulted in rapid bacterial clearance that was complete 7 days after challenge (Fig. 3A). The results suggest that factors other than cells from a single lymphoid organ, such as circulating or mucosal antibodies, also contribute to the clearance of bacteria in immune animals.

The role of circulating antibody in protection against infection was assessed by passive transfer of serum from convalescent immune animals. In comparison with serum from control naive mice, immune serum injected intravenously before challenge reduced the viable *B. pertussis* counts in the lungs of recipient mice by approximately 1 log unit soon after challenge (Fig. 3B).

MHC restriction of protective T cells. Since our cell purification techniques employed depletion rather than positive enrichment, it was important to rule out the possible contribution of non-MHC-restricted effector cells such as macrophages, NK cells, or TCR- γ/δ^+ T cells that may have been present in our T-cell preparations. The availability of B10.AQR congenic mice compatible with CBA (*H-2^k*) mice at the MHC class II loci enabled us to examine the MHC restriction of protection with CBA mice as recipients of purified T cells (Fig. 4). Adoptive transfer of immune T cells from syngeneic CBA donors conferred a high level of immunity, as seen by the undetectable level of bacteria in recipients 14 days after challenge. In contrast, injection of allogeneic immune T cells from BALB/c (*H-2^d*) mice into CBA (*H-2^k*) mice failed to transfer immunity, and there was some evidence that it decreased the rate of clearance over that in the control group. The bacterial counts in lungs were higher in the recipients of BALB/c cells 14 and 21 days after challenge than in irradiated mice that did not receive T cells. After transfer of immune T cells from B10.AQR congenic mice (compatible with the CBA recipients at the class II loci but not at the class I loci), bacterial counts were high on day 14 but undetectable by day 21. Although these findings suggest that compatibility at the class II locus alone is not sufficient for optimum transfer of immunity with T cells, the outcome of this experiment is complicated by the possible adverse contribution of graft-versus-host disease, which is likely to have resulted from injection of class I-mismatched B10.AQR T cells into irradiated CBA recipients. Indeed, graft-versus-host disease may also explain the apparent exacerbated pertussis seen in CBA mice after inoculation of fully MHC-incompatible BALB/c T cells compared with that in the control group. Nevertheless, the results do indicate that protection is mediated by MHC-restricted T cells and that a class II-restricted response has a significant contribution.

CD4⁺ T cells mediate protection. The phenotype of the T

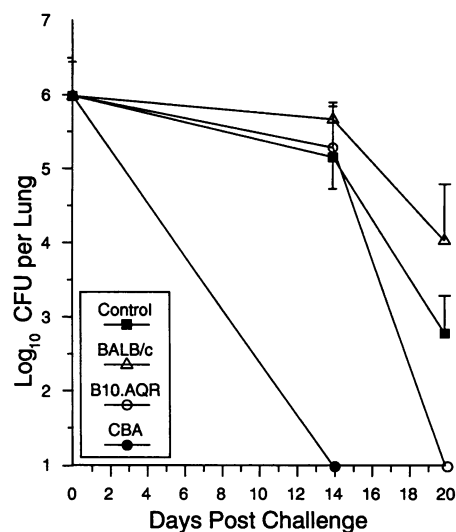


FIG. 4. Adoptive transfer of protection with MHC-restricted immune T cells. Sublethally irradiated CBA mice were injected intravenously with one spleen equivalent of T cells (2×10^7) from convalescent CBA (*H-2^k*), BALB/c (*H-2^d*), or congenic B10.AQR ($D^qI-A^*I-E^*K^d$) mice or from normal nonimmune CBA mice (controls). Recipients were aerosol challenged, and the numbers of CFU were estimated at intervals after infection. Results are mean (\pm standard error) CFU estimated for individual lungs from four mice per group at each time point.

cells involved in protection against respiratory pertussis infection was examined by adoptive transfer of purified immune CD4⁺ or CD8⁺ T-cell subpopulations. The CD4⁺ and CD8⁺ T cells were purified from spleen cells by B-cell, macrophage, and CD8⁺ or CD4⁺ depletion, respectively, and FACScan analysis revealed that they were 75 to 80% pure with less than 2% contamination with the reciprocal subset and less than 5% B cells. A positive role for CD4⁺ was clearly demonstrated in the experiments shown in Fig. 5A. Transfer of immune CD4⁺ T cells followed by respiratory *B. pertussis* challenge resulted in complete bacterial clearance by day 20. In contrast, CD8⁺ T cells did not confer any protection; on days 14 and 21, the viable counts in the lung were 10^6 to 10^7 , approximately 10-fold higher than those in the control group injected with nonimmune T cells from naive BALB/c mice (Fig. 5B). One of the animals injected with CD8⁺ cells in this experiment and two in a second experiment (data not shown) died 16 to 20 days after challenge. Transfer of an equivalent number (10^7) of unseparated immune T cells reduced the bacterial load 100-fold by day 21 (Fig. 5B). The less significant clearance seen with the T cells in this experiment compared with those shown in Fig. 3A and 4 may be accounted for by a reduction in the total numbers of cells transferred in this experiment (designed so that equal numbers of cells were injected into each mouse). Furthermore, the possible negative influence of the CD8⁺ T cells may also have contributed to reduced clearance with total T cells when compared with that seen in mice injected with the purified CD4⁺ T-cell population (Fig. 5A).

Further evidence for the protective role of antigen-specific CD4⁺ T cells was provided by the demonstration that a short-term T-cell line, BPF48, specific for FHA, also conferred a high level of immunity against aerosol challenge (Fig. 5A). The T-cell line was generated from the spleen cells of a convalescent mouse taken 8 weeks after respiratory

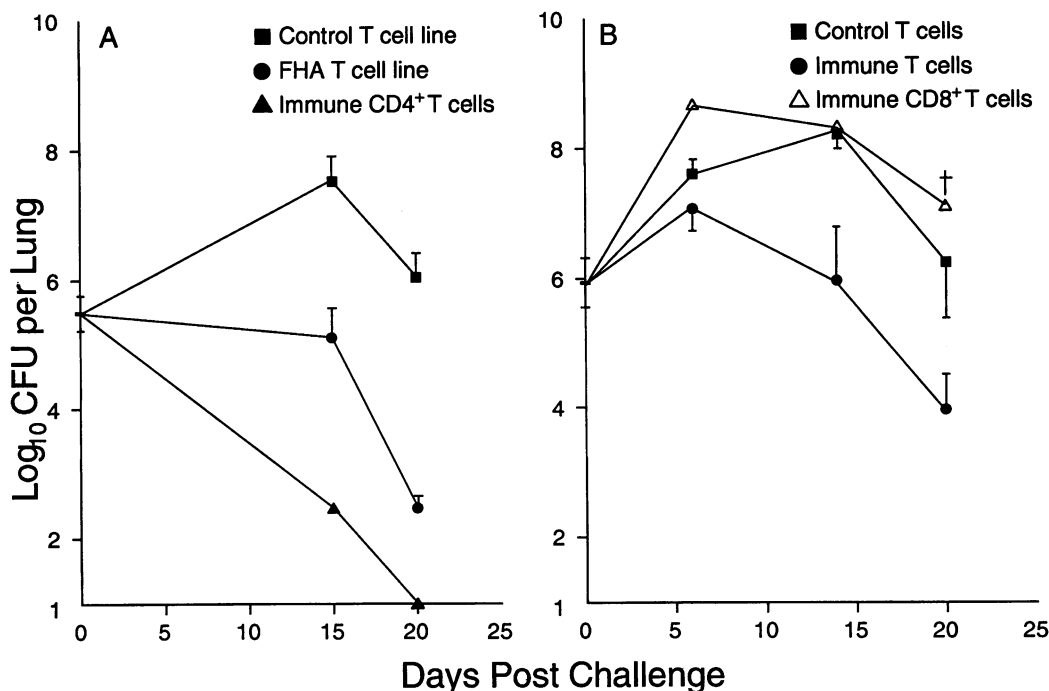


FIG. 5. Adoptive transfer of protection with CD4⁺ T cells from immune mice and with a CD4⁺ FHA-specific T-cell line. Sublethally irradiated BALB/c recipients were injected with the following: (A) immune CD4⁺ T cells purified by negative enrichment from spleen cells of convalescent mice, an FHA-specific T-cell line, BPF48 (65% CD4⁺ T cells, 12% CD8⁺ T cells, and 8% Ig⁺ cells), or a control CD4⁺ T-cell line specific for poliovirus; (B) purified T cells or CD8⁺ T cells from immune mice or T cells from control mice. All recipients received an equal number of cells (10⁷) and were aerosol infected; the numbers of CFU were estimated at intervals after infection. Results are mean (± standard error) CFU estimated for individual lungs from four mice per group at each time point.

infection by *in vitro* stimulation with FHA for 5 days followed by reculture in medium with feeder cells for 7 days. Phenotypic analysis of the line revealed that it included 65% CD4⁺, 12% CD8⁺, and 5% surface Ig⁺ cells. Although this and other *B. pertussis*-specific T-cell lines and clones have been maintained in culture for prolonged periods, a short-term line was deliberately chosen for transfer to maintain a broader antigen specificity and to increase the possibility of retaining homing receptors. In comparison with a control CD4⁺ T-cell line specific for an unrelated poliovirus antigen, transfer of the FHA-specific T-cell line resulted in a substantial reduction in the lung counts 14 and 21 days after pertussis challenge.

Antibody responses after *B. pertussis* infection. Having established a role for T cells in the protective immune response to *B. pertussis*, we attempted to define the mechanism of T-cell-mediated clearance by examining antibody responses in mice after primary or secondary challenge and after CD4⁺ T-cell transfer. Circulating serum antibodies to PT, FHA, pertactin, or a *B. pertussis* sonic extract were almost undetectable by the ELISA up to 4 weeks after primary infection in normal BALB/c mice (Fig. 6A). At 6 weeks, when the animal would have already cleared the infection (Fig. 1), 6 U of serum antibodies to PT per ml were demonstrated; the amounts increased steadily thereafter (Fig. 6A). A weak antibody response to FHA was detected at 6, 8, and 12 weeks postinfection, but levels of antibody to pertactin and a *B. pertussis* sonic extract in serum remained below detectable levels up to 12 weeks after infection. The lack of a detectable IgG response to the *B. pertussis* sonic extract probably reflects the overall low levels of antipertus-

sis antibody in convalescent-phase serum and the relatively small proportion of individual components (e.g., PT and FHA) in the sonic extract. IgM antibodies to *B. pertussis* were also undetectable in serum throughout the period of study (data not shown). By comparison, immunization with the whole-cell vaccine (two doses) induced approximately 100 U of IgG against FHA, PT, pertactin, and *B. pertussis* sonic extract per ml in serum (data not shown).

The generation of antibodies to pertussis in the lung was also examined at intervals after infection. Specific IgA antibody levels were found in lung homogenates (Table 1) and lung lavage samples, but IgA antibodies were undetectable in serum (data not shown). PT-specific IgA antibodies were present in the lungs 14 days after infection, but IgA antibodies to a *B. pertussis* sonic extract only reached detectable levels 2 weeks later (Table 1). In contrast, enhanced lung IgA antibodies were demonstrated against PT and a *B. pertussis* sonic extract 14 days after rechallenge of convalescent mice.

The serum of mice that had been challenged after transfer of murine CD4⁺ T cells was also examined for anti-*B. pertussis* antibodies. Twenty days after challenge, when the mice had cleared the infection, we failed to detect in serum any IgG, IgM, or IgA antibodies to a *B. pertussis* sonic extract, PT, FHA, or pertactin (Table 1; data not shown).

***B. pertussis*-specific T-cell responses in immune mice.** In contrast to the low levels and late appearance of anti-*B. pertussis* antibodies, strong T-cell responses (detected by *in vitro* proliferation and lymphokine production) were consistently demonstrated 2, 4, 6, 8, and 12 weeks after primary infection (Fig. 6B). The T cells generated by infection

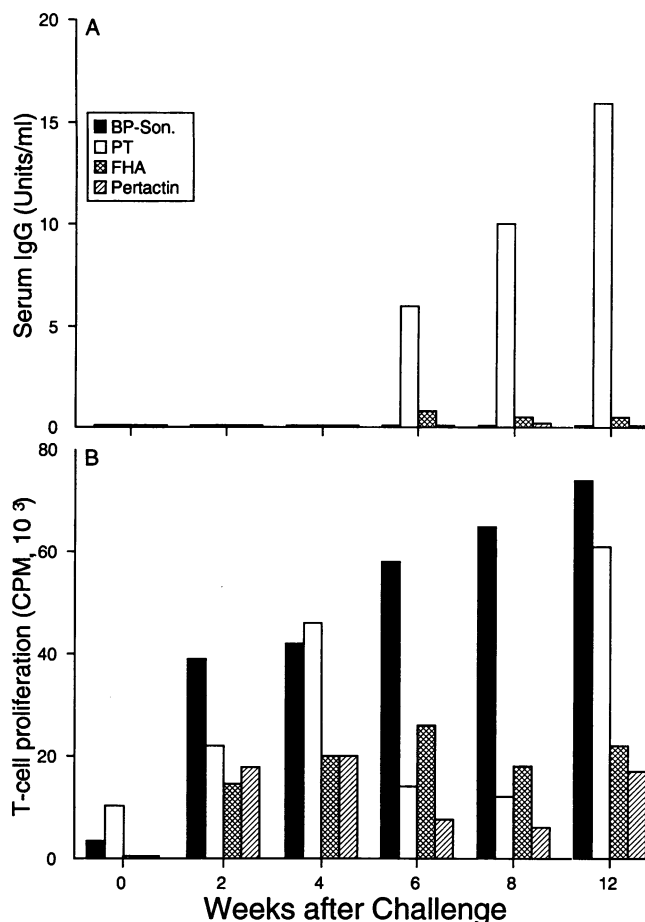


FIG. 6. Pertussis-specific serum antibody (A) and T-cell (B) responses in normal BALB/c mice during the course of a primary respiratory infection. Serum antibody responses were detected by ELISA and are given as arbitrary units with reference to a standard serum. Results are mean values for four mice at each time point (standard errors were <10%). BP-Son, *B. pertussis* sonic extract.

responded to FHA, pertactin, PT, agglutinogens 2 and 3, and a soluble pertussis sonic extract. The weak proliferative response to PT and, to a lesser extent, to the *B. pertussis* sonic extract in spleen cells from control animals before

infection (day 0 in Fig. 6B) can be accounted for by the mitogenic activity of PT for murine T cells. Although we have used a dose of PT (0.2 µg/ml) that is at the lower end of the mitogenic range, the PT still stimulated a degree of proliferation in naive cells. However, the significantly greater responses with cells from infected animals suggest that the proliferative T-cell responses to PT and *B. pertussis* sonic extract were largely antigen specific. This was confirmed by using a genetically detoxified PT that was devoid of mitogenic activity at the dose used (data not shown).

MHC restriction of the T-cell response. The MHC restriction of the in vitro response of immune T cells to *B. pertussis* antigens was studied by using APC from syngeneic, allogenic, and congenic donors (Table 2). Highly purified T cells (98%) from convalescent BALB/c mice responded to heat-killed *B. pertussis* cells and to PT presented by APC from syngeneic mice but not to those presented by APC from allogenic CBA mice. The lack of response of the purified BALB/c T cells to concanavalin A (ConA) and lipopolysaccharide in the absence of added accessory cells confirms the high purity of this T-cell preparation. The proliferation of BALB/c T cells to ConA in the presence of irradiated spleen cells from CBA mice reflects the non-MHC-restricted role for accessory cells in a mitogenic response. In a second experiment in which the T cells were not as highly purified, a weak response to ConA was detected without the addition of accessory cells. However, in both experiments the antigen-specific T-cell response was MHC class II restricted. The purified T cells from convalescent CBA mice responded to a *B. pertussis* sonic extract, FHA, and PT presented by APC from syngeneic or Ia region-compatible congenic B10.AQR mice but not those presented by APC from allogenic BALB/c mice. Similarly, a positive antigen-specific T-cell response was demonstrated with B10.AQR T cells in the presence of APC that were syngeneic and I-A and I-E (CBA *H-2^d*) region compatible. These results demonstrate that the in vitro *B. pertussis*-specific T-cell responses were MHC class II restricted.

Lymphokine secretion by pertussis-specific T cells. In a further attempt to define the nature of the T-cell responses generated by *B. pertussis* infection, we examined the phenotypes and lymphokine secretion patterns of in vitro antigen-stimulated spleen cells from immune mice. Spleen cells derived from convalescent animals 6 weeks after aerosol challenge responded in vitro to heat-killed *B. pertussis*, PT, FHA, and pertactin by the production of lymphokines (IL-2

TABLE 1. Anti-*B. pertussis* serum IgG and lung IgA antibodies^a after challenge of normal or convalescent mice or recipients of immune CD4⁺ T cells

Treatment group	Day postchallenge	Serum IgG (U/ml)			Lung IgA (titer)		
		BP	PT	FHA	BP	PT	FHA
Normal	0	<0.01	<0.01	<0.01	<5	<5	<5
	14	<0.01	<0.01	0.01	<5	25 ± 4	<5
	28	0.02 ± 0.01	0.01	0.16 ± 0.13	5 ± 2	24 ± 8	<5
Convalescent	0	0.04 ± 0.02	9.2 ± 4	0.25 ± 0.18	5	47 ± 14	<5
	14	0.08 ± 0.03	23 ± 18	0.20 ± 0.15	185 ± 79	80 ± 28	<5
CD4 ⁺ T-cell transfer	0	<0.01	<0.01	<0.01		ND	
	14	<0.01	<0.01	<0.01			
	20	<0.01	0.10	0.01			

^a Antibodies to *B. pertussis* sonic extract (BP), PT, and FHA were measured by ELISA. Results are means ± standard errors for four mice at each time point. Results for IgG in serum are given after reference to standard anti-*B. pertussis* serum raised in mice against the whole-cell vaccine. IgA levels in the lung are given as reciprocal endpoint titers after dilution of lung homogenates prepared in 1 ml of PBS. ND, not determined.

TABLE 2. MHC class II-restricted in vitro T-cell response to *B. pertussis*

T cells ^a	APC ^b	Proliferative response (cpm, 10 ³) to ^c :						
		No antigen	BP	FHA	PT	LPS	ConA	
Expt 1 BALB/c	None	0.1 ± 0.1	0.1 ± 0.1	ND	0.1	0.1 ± 0.1	0.3 ± 0.1	
	CBA (kkkk)	0.1	0.1	ND	0.1 ± 0.1	0.2 ± 0.1	134.9 ± 9.8	
	BALB/c (dddd)	0.1	55.8 ± 6.2	ND	3.7 ± 0.2	0.8 ± 0.1	103.2 ± 8.6	
Expt 2	CBA	None	0.4 ± 0.1	0.8 ± 0.2	4.0 ± 0.1	0.5 ± 0.1	ND	12.1 ± 1.3
		CBA (kkkk)	0.9 ± 0.2	7.7 ± 0.9	3.8 ± 0.2	11.0 ± 1.6	ND	45.8 ± 7.2
		BALB/c (dddd)	0.6 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	5.0 ± 0.7	ND	10.3 ± 2.1
	B10.AQR	B10.AQR (qkkd)	1.7 ± 0.2	8.8 ± 0.6	4.2 ± 0.5	11.5 ± 1.4	ND	39.5 ± 6.4
		None	0.1 ± 0.1	1.4 ± 0.7	3.0 ± 0.1	0.4 ± 0.1	ND	13.4 ± 0.9
		CBA (kkkk)	0.7 ± 0.1	14.7 ± 1.8	6.5 ± 0.5	7.4 ± 0.9	ND	68.8 ± 8.5
		BALB/c (dddd)	1.2 ± 0.1	2.9 ± 0.4	1.0 ± 0.3	5.9 ± 1.2	ND	79.1 ± 7.4
		B10.AQR (qkkd)	0.5 ± 0.1	17.7 ± 2.1	9.7 ± 1.3	15.2 ± 2.1	ND	51.6 ± 4.3
		NIH (qqqq)	1.6 ± 0.1	2.0 ± 0.2	1.8 ± 0.2	4.7 ± 0.5	ND	63.3 ± 5.1

^a T cells were purified from spleen cells derived from immune mice 6 weeks after respiratory infection by depletion of B cells and macrophages on Ig-coated plastic dishes. The purified cell preparations were approximately 70% T cells and 5% B cells. In experiment 1 the spleen cells were passed through a nylon-wool column before panning; this resulted in a T-cell population that was 98% pure with less than 0.2% B cells. T cells (2 × 10⁵/ml) were cultured with APC and heat-killed bacteria (BP); purified *B. pertussis* antigen FHA, PT, or LPS; or ConA.

^b Irradiated spleen cells (2 × 10⁶/ml) from naive mice of different *H-2* haplotypes (K, I-A, I-E, and D loci are given within parentheses) were used as sources of APC.

^c Results are means ± standard errors for triplicate cultures of cells pooled from four to six mice. ND, not determined.

or IL-4) that support the growth of the CTLL cell line (Table 3). The addition of an MAb to IL-4, which completely inhibited the response of the CTLL line to IL-4, did not reduce the proliferation of the CTLL line in the presence of supernatants from the antigen-stimulated spleen cells, suggesting that the lymphokine detected was predominantly IL-2. We also failed to detect IL-4 with the immunoassay (Table 4). However, significant levels of IFN- γ were demonstrated in supernatants from immune spleen cells stimulated with *B. pertussis* antigen in vitro (Table 4).

Purified T cells or CD4⁺ T cells from spleens of immune mice also responded with IL-2 production (Table 5) and proliferation (data not shown) to a range of *B. pertussis* antigens. In contrast, CD8⁺ T cells failed to proliferate or secrete IL-2 in response to heat-killed *B. pertussis*, FHA, or

pertactin. The ability of purified CD8⁺ T cells to produce IL-2 in response to ConA and, to a lesser extent, active PT demonstrates that the mitogen reactivity of these cells is intact. These results suggest that the T cells induced by natural infection are largely confined to CD4⁺ T cells that secrete IL-2 and IFN- γ but not IL-4.

DISCUSSION

The results of this study provide the first evidence of a direct role for T cells in the development of acquired resistance to *B. pertussis*. Using a murine respiratory infection model, we demonstrated by adoptive transfer that CD4⁺ T cells can mediate bacterial clearance in the absence of a detectable serum antibody response. Furthermore, the predominant T cells induced by infection were CD4⁺ T cells that secreted IL-2 and IFN- γ . Although our findings do not rule out T-cell helper function for a mucosal IgA response in the lung, they do suggest that cellular immune responses mediated by Th1 cells play a critical role in protective immunity to pertussis generated by respiratory infection in mice.

The importance of T cells in immunity to a variety of

TABLE 3. Secretion of IL-2 and IL-4 by antigen-stimulated spleen cells from *B. pertussis*-immune mice after in vitro antigen stimulation^a

Supernatant or lymphokine	CTLL proliferation (cpm, 10 ³) ^b	
	No antibody	Anti-IL-4
Spleen	1.6 ± 0.4	1.4 ± 0.2
Spleen + BP (10 ⁶ /ml)	12.5 ± 1.5	17.3 ± 2.1
Spleen + FHA (1 μ g/ml)	8.9 ± 1.2	9.9 ± 1.3
Spleen + pertactin (5 μ g/ml)	15.6 ± 2.0	17.2 ± 2.4
Spleen + PT (0.2 μ g/ml)	11.6 ± 1.7	14.4 ± 2.2
rIL-2 (1.0 IU/ml)	21.5 ± 3.0	24.8 ± 4.1
rIL-2 (5.0 IU/ml)	36.2 ± 5.6	41.2 ± 3.5
IL-4 (50 U/ml)	11.9 ± 0.8	0.8 ± 0.1
IL-4 (250 U/ml)	24.9 ± 2.5	1.2 ± 0.2

^a Spleen cells (2 × 10⁶/ml) pooled from four convalescent immune mice (6 weeks after infection) were stimulated in vitro with heat-killed *B. pertussis* (BP) or *B. pertussis* antigens, and the supernatants taken 24 h later were tested for their ability to support the proliferation of the CTLL-2 cell line in the presence or absence of 10 μ g of 11B11 (anti-IL-4) MAb per ml. Recombinant human IL-2 (rIL-2) and murine IL-4 were added directly to CTLL cells at the indicated final concentrations.

^b Results are means ± standard errors for triplicate cultures.

TABLE 4. Production of cytokines by spleen cells from immune mice in response to *B. pertussis* antigen^a

Mouse no.	IFN- γ (IU/ml)		IL-4 (U/ml)	
	No antigen	+BP	No antigen	+BP
1	<1	70	<5	<5
2	<1	24.5	<5	<5
3	<1	66	<5	<5
4	<1	90	<5	<5

^a Spleen cells from individual convalescent immune mice (taken 6 weeks after infection) were cultured without antigen or with heat-killed *B. pertussis* (BP). Supernatants were removed after 72 h and assayed in duplicate for IFN- γ by the immunoradiometric assay and IL-4 by the ELISA. *B. pertussis*-stimulated spleen cells from normal nonimmune mice produced no detectable IFN- γ .

TABLE 5. Mediation of in vitro antigen-specific IL-2 response of *B. pertussis*-immune spleen cells by CD4⁺ T cells

T cells ^a	IL-2 (IU/ml) ^b					
	No antigen	BP	FHA	Pertactin	PT	ConA
Total	<0.1	6.1 ± 0.9	1.6 ± 0.2	5.1 ± 0.6	3.9 ± 0.5	6.3 ± 0.7
CD8 ⁺	<0.1	0.1 ± 0.1	<0.1	<0.1	0.3 ± 0.1	2.5 ± 0.4
CD4 ⁺	<0.1	5.5 ± 0.1	3.0 ± 0.4	2.1 ± 0.3	2.2 ± 0.3	14.0 ± 2.1
Line BPF48	<0.1	3.0 ± 0.4	1.5 ± 0.1	<0.1	ND	ND

^a Total T cells or CD4⁺- or CD8⁺-enriched subpopulations (2×10^5 /ml) purified from the spleens of six convalescent immune mice or a short term CD4⁺ T-cell line, BPF48, specific for FHA (1×10^5 /ml) were cultured with APC (2×10^6 /ml; autologous irradiated spleen cells) and antigens or mitogens. Supernatants were removed after 24 h and assayed for IL-2 by CTLL proliferation in the presence of anti-IL-4 MAb 11B11. BP, *B. pertussis* cells.

^b Results are means ± standard errors for triplicate assays after reference to a standard curve with recombinant IL-2. ND, not determined.

infectious pathogens is well documented (23, 31, 35). CD4⁺ T cells have been predominantly implicated in providing helper function for humoral immune response to extracellular pathogens (7, 18, 21, 35), whereas CD8⁺ cytotoxic T lymphocytes (CTL) have been shown to be capable of controlling diseases resulting from infection with viruses (31, 57) and intracellular bacteria (23) and parasites (62). However, CD4⁺ T cells can also mediate cellular immunity against intracellular pathogens through the lysis of infected cells (24, 37, 59) or by releasing cytokines that stimulate antimicrobial activity of neutrophils or macrophages (26, 50, 56). Since until recently *B. pertussis* was considered to be a noninvasive bacterium, it has been generally assumed that protective immunity to this pathogen, like that to other extracellular pathogens, is exclusively mediated by antibodies (18–21, 60). Circumstantial evidence for the role of T cells in immunity to pertussis is provided by the suggestion of an in vivo intracellular state for *B. pertussis*, which would escape immune defenses mediated by humoral responses (8, 11, 16, 28, 48). However, there has been little attempt to establish a direct role of T cells in protective immunity to pertussis.

In the present study we used the murine aerosol infection model to examine the mechanism of acquired resistance generated by respiratory infection. Although the intracerebral challenge model has been accepted as the laboratory correlate for vaccine-induced protection in humans and has been used extensively to study the role of antipertussis antibody in active and passive immunization experiments (43), its relevance to studies of the mechanism of protective immunity is questionable. The lack of virulence of fresh clinical isolates of *B. pertussis* by this route and the possible differences in host bacterial interaction in the brain and respiratory tract are serious concerns (25, 38, 47). Therefore, we use the nonlethal mouse aerosol infection model, in which many of the parameters of the infection are similar to those observed in children (41, 45, 55). Our rationale for the study of donors that had had previous *B. pertussis* infection rather than immunization is based on the observation that the strongest and most persistent immunity in humans (17) and mice (3, 5) is generated by respiratory infection; therefore, our approach provides a sound basis for dissecting the protective immune response and for designing improved vaccination strategies. Natural immunity induced by infection may prevent respiratory colonization, whereas that generated by current pertussis vaccines may mainly protect against toxin-induced disease (17).

The importance of T cells in the resolution of *B. pertussis* infection is evident from our demonstration that T-cell-deficient athymic mice fail to clear the bacteria after aerosol challenge. Furthermore, the persistent infection in suble-

thally irradiated mice and in AIDS patients suggests that pulmonary resistance is compromised in an immunosuppressed host. In normal naive adult mice, the resident host defenses, such as mucociliary clearance or the bactericidal activity of resident pulmonary phagocytic cells, are also unable to prevent the infection. However, naive immunocompetent mice with acquired immunity generated during a respiratory infection appear to be eventually capable of completely eliminating the organism, as shown by the absence of detectable CFU in the lungs 5 weeks after challenge. In an examination of humoral and cell-mediated responses generated during infection, we demonstrated a strong and persistent T-cell response throughout the course of infection but insignificant levels of anti-pertussis IgG, IgM, or IgA antibodies in the serum up to 4 weeks after primary exposure. However, we did detect IgA antibodies to PT in the lungs 14 days after challenge, suggesting that mucosal antibodies are also involved in the clearance of a primary pertussis infection.

Adoptive transfer experiments provided further evidence of a direct role for immune T cells in the development of acquired immunity to pertussis. Transfer of unseparated spleen cells from convalescent mice into nude or sublethally irradiated recipients resulted in complete bacterial clearance as early as 14 days after challenge. In contrast, passive transfer of convalescent-phase serum (which contained anti-PT IgG but low or undetectable levels of antibodies to FHA and pertactin) when compared with normal mouse serum reduced the bacterial counts by only 1 log unit during early stages of infection. However, as suggested by Kimura et al. (25), it is possible that a delay of 24 h between antibody administration and challenge could have resulted in a more dramatic effect. Depletion of T cells from the immune spleen cell population reduces their protective capacity but not to the level seen after transfer of normal nonimmune cells. Furthermore, depletion of B cells also delayed clearance, suggesting that primed B cells contribute to the protective effect of immune spleen cells, possibly through the development of a local amnestic antibody response after migration to the lungs (51).

A detailed in vivo and in vitro analysis of the pertussis-specific T cells suggested that MHC class II-restricted CD4⁺ T cells with a lymphokine-secreting profile characteristic of Th1 cells play a central role in immunity to pertussis. Purified T cells (B cells and macrophage-depleted spleen cells) from immune mice transferred protection to syngeneic recipients but not allogeneic recipients. Although the transfer of immunity was reduced in congenic mice mismatched at the class I loci, these experiments were complicated by the graft-versus-host disease, which is likely to have occurred after injection of MHC-incompatible cells into irradiated

recipients. However, when taken together with MHC restriction analysis of pertussis-specific responses in vitro, the data suggest that MHC class II-restricted T cells are primarily involved. This conclusion is compatible with the demonstration that purified CD4⁺ T cells, but not CD8⁺ T cells, conferred a high level of protection. Indeed, the rate of clearance after transfer of CD4⁺ T cells was quicker than that with the equivalent number of unseparated T cells, suggesting that the CD8⁺ T cells have a negative role. This was confirmed in two separate experiments in which transfer of purified CD8⁺ cells resulted in bacterial counts that were elevated relative to those seen with nonimmune T cells and in the deaths of a number of animals.

The significant reduction in bacterial counts in lungs after transfer of a short-term FHA-specific T-cell line provides further evidence for the protective role of CD4⁺ T cells. The lymphokine secretion pattern of this T-cell line and those of the majority of T-cell clones generated from convalescent mice were characteristic of Th1 cells (5). Furthermore, in vitro antigen stimulation of spleen cells from convalescent mice resulted in production of IL-2 and IFN- γ but no detectable IL-4. However, the detection of cytokines secreted by Th2 cells may require in vitro restimulation (7). Indeed, we have found that a minority of *B. pertussis*-specific CD4⁺ T-cell clones generated from infected mice do produce IL-4 (5). Nevertheless, our results suggest that the predominant T cells induced by infection are Th1 cells and are in agreement with the recent demonstration that *B. pertussis*-specific CD4⁺ T-cell clones from previously infected humans secrete IL-2 and IFN- γ , but little or no IL-4, after in vitro antigen stimulation (40).

The demonstration of clearance of *B. pertussis* from the lungs of mice in the absence of a significant serum antibody response during a primary infection and after transfer of CD4⁺ T cells is compatible with the role of Th1 cells in classical cell-mediated immunity. Although we cannot rule out the possibility that the T cells provided helper activity for a mucosal IgA response in the lung, the synthesis of IgA appears to be primarily under the control of Th2 cells. IL-4 has been shown to be involved in B-cell IgA isotype switch from IgM precursors, whereas IL-5 and IL-6 stimulate IgA synthesis by IgA-committed B cells (6, 14, 27). However, IL-2 alone or in combination with IFN- γ or transforming growth factor β has also been implicated in stimulating IgA production by acting at the pre- or postswitch level (12, 14, 36). Our findings of enhanced anti-*B. pertussis* IgA levels in the lungs after challenge of convalescent mice is suggestive of a role of mucosal antibodies in protective immunity to pertussis. The function of distinct T-cell subsets and their secreted cytokines in the regulation of this response remains to be established.

Alternatively, there are a number of possible mechanisms of non-antibody-mediated cellular immunity to pertussis. Th1 cells have been shown to play a role in immunity to intracellular pathogens through their cytotoxic function against the infected cells (24, 59). Although *B. pertussis* has been considered to be a noninvasive pathogen and replicates on the surface of epithelial cells, recent evidence suggests that it can enter and survive within mammalian cells, including pulmonary macrophages. Furthermore, CD4⁺ T-cell clones derived from immune humans have been shown to display in vitro cytotoxic activity against *B. pertussis*-pulsed target cells (40). Therefore, CD4⁺ CTL could, through the release of organisms for attack by other immune effector mechanisms, help to destroy this intracellular reservoir of bacteria and thereby limit the course of the infection. *B.*

pertussis-specific CD4⁺ T cells could also stimulate the antibacterial activity of phagocytic cells. The cytokines IFN- γ and lymphotoxin, which are released by Th1 cells, have been shown to mediate protection against other infectious diseases through the stimulation, recruitment, and activation of macrophages and neutrophils (26, 50). However, cellular responses mediated by Th1 cells have been largely associated with protection against intracellular bacteria (24, 37). It remains to be defined whether the Th1-like responses demonstrated in the present study are exclusively directed against the putative intracellular phase of pertussis or also involved in clearance of the predominantly extracellular organisms.

Our findings do not obviate an antibody-mediated mechanism of immunity to pertussis generated by immunization. However, in the present murine model of natural immunity the capacity to generate a rapid and consistent CD4⁺ T-cell response appears to be a major factor in the development of host resistance to a *B. pertussis* respiratory infection. Since the nonlethal respiratory infection of adult mice with *B. pertussis* is a model for colonization and not for disease, it cannot be concluded that the same immune effector mechanisms operate in protection against the human disease. Nevertheless, when taken together with the demonstration of *B. pertussis*-specific T-cell induction and lack of serological correlates of protection in humans (2, 15, 40), these findings suggest that cell-mediated immunity also plays a role in the prevention or control of pertussis in humans. Therefore, future development and evaluation of pertussis vaccines should be orientated toward stimulation and testing of cellular as well as humoral immunity.

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