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Proliferative Responses and Gamma Interferon and Tumor Necrosis Factor Production by Lymphocytes Isolated from Tracheobroncheal Lymph Nodes and Spleens of Mice Aerosol Infected with *Bordetella pertussis*

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A group of mice was aerosol infected with live, virulent *Bordetella pertussis* bacteria. During a period of 7 weeks following the infection, with intervals of 1 week, lymphocytes were isolated from the tracheobroncheal lymph nodes (TBL) and the spleens (SPL) of the infected mice. The in vitro proliferative responses as well as the gamma interferon and tumor necrosis factor production levels of the isolated lymphocytes in response to stimulation with whole killed *B. pertussis* bacteria were measured as parameters for cell-mediated immunity (CMI). The course of the infection was monitored by counting of CFU in the lungs of the mice. Moreover, antibody responses in serum against a range of *B. pertussis* antigens were assessed. The results showed that a vigorous proliferative response of the TBL and SPL to stimulation with whole killed *B. pertussis* bacteria was induced by the infection. The proliferative response of the TBL was significantly higher than the response of the SPL. The proliferative responses were maximal 3 to 4 weeks after the infection and were paralleled by in vitro gamma interferon and tumor necrosis factor production upon specific stimulation. The development of the CMI was observed simultaneously with the clearance of the infection from the lungs. Antibody responses became measurable in the sera only after the infection was cleared. A specific CMI against pertussis toxin, the filamentous hemagglutinin, the 69-kDa outer membrane protein, and the agglutinogens 2 and 3, antigens which are under consideration for inclusion in future acellular pertussis vaccines, was successfully demonstrated in mice 3 weeks after the infection.

Bordetella pertussis infects the respiratory tract of the human host and causes whooping cough in children. The nature of the immunity against *B. pertussis* infection and disease is poorly understood. In animal models of infection, antibodies are known to play a role in protection (15, 30, 32, 35), and immunization with pertussis vaccine induces an antibody response against several *B. pertussis* antigens in mice and humans (26, 36, 37). In recent years, however, the role of cell-mediated immunity (CMI) in protection against pertussis has drawn still more attention. A recent Swedish field trial has failed to show any correlation between protection against clinical pertussis and the levels of antibodies against pertussis toxin (PT) in postvaccination sera from infants vaccinated with acellular pertussis vaccine (1). Furthermore, peripheral T cells reactive with the *B. pertussis* antigens PT, the filamentous hemagglutinin (FHA), and the 69-kDa outer membrane protein antigen (OMP) have been demonstrated in human pertussis convalescents (6, 11, 38, 39). For these reasons and others, a role for CMI following pertussis infection or vaccination may be hypothesized. CMI may act on different levels, e.g., through (i) T-helper cells to facilitate antibody synthesis (40), (ii) activation of other cell types (e.g., macrophages and neutrophils [12]), and (iii) the action of cytotoxic T cells, since *B. pertussis* organisms are reported to invade other cell types (8, 17) and survive intracellularly in macrophages (33).

Experimental *B. pertussis* infection of mice is located in the upper respiratory tract and the lungs, in contrast to natural infection in humans, which is almost exclusively located in the ciliated epithelium of the upper respiratory tract and only rarely reaches the lungs (15). The tracheobroncheal lymph nodes (TBL) drain the lungs of mice, and it has been shown that lung macrophages actively transport antigen from the lung lobes to the TBL (13). T-cell reactivity of lymphocytes from the TBL to inhaled antigen has also been demonstrated elsewhere (23).

In the present study, we have investigated the CMI against *B. pertussis* antigens in mice aerosol infected with virulent *B. pertussis* organisms. As parameters for CMI, T-lymphocyte proliferative responses in vitro and gamma interferon (IFN-γ) as well as tumor necrosis factor (TNF) production were chosen. Lymphocytes from infected mice were isolated from the TBL, representing the regional immune response of the lungs, and from the spleen (SPL), representing the systemic immune response. The proliferative responses and in vitro cytokine production in response to stimulation with whole *B. pertussis* bacteria (WB) were studied from 1 to 7 weeks after the infection. The results were compared with the progression of the infection in the lungs and the development of a specific serum antibody response. Furthermore, the proliferative responses and cytokine production to purified *B. pertussis* antigens were studied 3 weeks following the infection.

MATERIALS AND METHODS

Mice. C57BL/6j mice were purchased from Bomholtgård Breeding and Research Centre Ltd., Ry, Denmark. Female mice, 4 to 8 weeks old, were used in all experiments.

Growth of bacteria. Freeze-dried *B. pertussis* bacteria (vaccine strain 3843) were diluted in saline and plated on

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Bordet-Gengou plates. The plates were incubated at 35°C for 72 h. The bacterial colonies were transferred to shaker bottles containing Stainer Scholte medium supplemented with cyclodextrin (1 mg/ml) and incubated for 24 h at 35°C. The bacterial concentration at the end of the incubation period was approximately 35 × 10^9 bacteria per ml. The bacterial suspensions were diluted in phosphate-buffered saline (PBS) and then used for aerosol infection.

Aerosol infection. Groups of mice were exposed to an aerosol of *B. pertussis* 3843, generated from a suspension of 10 × 10^9 bacteria per ml of PBS that was supplemented with 1% Casamino Acids (pH 7.4), for 30 min. The viability of the bacteria was ensured by plating on Bordet-Gengou plates containing 25 μg of cephalexin per ml.

Antigens. (i) WB antigens. The Danish whole-cell pertussis vaccine, consisting of killed, phase 1 *B. pertussis* bacteria (16 × 10^9 cells per ml) from vaccine strains 3803, 3825, 3843, and 3860 and containing 0.1 mg of thiomersal per ml, was washed twice in saline before use as an antigen for in vitro stimulation.

(ii) Purified antigens. PT, PT detoxified by formaldehyde (dPT-F), FHA, and the 69-kDa OMP were a generous gift of Carine Capiau, SmithKline Beecham, Rixensart, Belgium (4, 5). PT detoxified by hydrogen peroxide (dPT-H) (34) was a generous gift of the North American Vaccine Corporation, Washington, D.C. PT detoxified by recombinant techniques (dPT-r) was a generous gift of Rino Rappuoli, Sclavo Research Center, Siena, Italy (25). Purified *B. pertussis* agglutinogens 2 (Agg 2) and 3 (Agg 3) were a generous gift of Andrew Robinson, Public Health Laboratory Service, Porton Down, United Kingdom. The purity of all antigen preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. The 80-kDa antigen was purified by gel filtration, ion-exchange, and adsorption chromatographies (unpublished data).

Enumeration of CFU in lungs. Mice were killed with CO_2, and their lungs were removed. The lungs of individual mice were homogenized in PBS with a mortar. Dilutions of lung homogenates were plated on Bordet-Gengou plates containing cephalexin. The number of CFU was counted after 4 to 7 days of incubation. Colonies of *B. pertussis* were not found in mice which had not been subjected to experimental infection.

Lymphocyte stimulation test. TBL and SPL were removed from infected and noninfected mice, minced with a scalpel blade, and gently pressed through a steel wire mesh (14). Lymphocytes from TBL and SPL were prepared in RPMI 1640 supplemented with 5 × 10^{-5} M 2-mercaptoethanol, 100 IU of penicillin per ml, 50 μg of streptomycin per ml, nonessential amino acids, and 1 mM glutamine (complete medium). The SPL were treated with 0.84% ammonium chloride in order to remove the erythrocytes. The cells were washed twice in complete medium and cultured in flat-bottom microtiter plates (Nunc, Roskilde, Denmark) (2 × 10^5 cells per well) in a volume of 200 μl of complete medium containing 0.5% (vol/vol) freshly prepared mouse serum without antigen (controls), with 1 μg of phytohemagglutinin (PHA) (Wellcome, Beckenham, United Kingdom) per ml as positive controls for cell reactivity or with antigen. All tests were carried out in triplicate. When necessary, lymphocytes from two or more mice were pooled. Cultures were incubated for 4 days at 37°C in an atmosphere of 5% CO_2 in humidified air. Twenty-two hours prior to harvesting, 1 μCi of [^{3}H]thymidine (Radiochemical Centre, Amersham, United Kingdom) was added to each well. The cells were harvested on fiberglass paper, and the incorporated radioactivity was measured in a liquid scintillation counter.

Quantitation of IFN-γ and TNF. TBL and SPL were isolated and cultured as described for the lymphocyte stimulation test. After 72 h of culture, the culture supernatants were harvested and stored at −20°C. The IFN-γ in the supernatants was quantitated by using the mouse IFN-γ enzyme-linked immunosorbent assay (ELISA) (Holland Biotechnology bv., Leiden, The Netherlands). The TNF was quantitated with the mouse TNF-α ELISA kit (Genzyme, Cambridge, Mass.). Both tests were used according to the manufacturers' prescriptions.

Identification of lymphocyte subsets. The T-cell receptors on the lymphocytes in culture were blocked by diluting the anti-CD4 (Tib 207) and anti-CD8 (Tib 105) monoclonal antibody (MAb) hybridoma supernatants 1:8 directly in the cultures, as described by Brett et al. (3) (dilution based on previous titrations). Addition of anti-CD4 or anti-CD8 had no effect on the proliferative response of PHA-activated cell cultures.

Quantitation of serum antibodies. Blood samples from mice were obtained by retroorbital plexus puncture. The blood was allowed to clot at room temperature, and the sera were isolated by centrifugation. ELISA was performed with polystyrene Maxisorb microtiter plates (Nunc). For the purified antigens, wells were coated overnight at 4°C with antigen in a carbonate buffer (pH 9.6) (PT, 0.4 μg/ml; FHA, 0.6 μg/ml; 69-kDa OMP, 1.9 μg/ml; Agg 2, 0.4 μg/ml; Agg 3, 0.4 μg/ml). For the WB, 50 μl of a suspension of *B. pertussis* 3843 (0.25 mg/ml in PBS) was added to each well, and the plates were dried overnight under an air fan at room temperature.

The plates were blocked with PBS containing 5% skim milk for 1 h at room temperature. The mouse serum samples were prediluted 1/20 in PBS containing 5% skim milk, diluted twofold in 12 wells, and incubated for 2 h at room temperature. Polyclonal antisera or MAbs were run as standards on each plate. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G plus immunoglobulin M (Pierce, Rockford, Ill.) was diluted 1/1,000 in PBS containing 5% skim milk, and the plates were incubated for 1 h at room temperature. Following this step, substrate buffer (0.7% citric acid and 1.2% Na_2HPO_4 [wt/vol; pH 5]) was applied to the wells for 5 min. Finally, a dye-substrate solution (0.4 mg of *O*-phenylenediamine per ml and 0.04% H_2O_2) was added and allowed to react for 30 min. Color development was stopped with 1 NH_2SO_4, and the A_{490} was read.

Statistical methods. Comparison of responses between groups of mice was done with the Mann-Whitney two-sample test. Comparison between TBL and SPL responses within groups of mice was done with the Wilcoxon signed-rank test. The levels of IFN-γ and TNF in culture supernatants at different time points following the experimental infection were compared by a one-way analysis of variance. Probability values below 0.05 were regarded as statistically significant.

RESULTS

Course of infection. A total of 40 mice were aerosol infected with virulent phase 1 *B. pertussis* bacteria. At 1 to 7 weeks following the infection, with intervals of 1 week, groups of five mice were sacrificed, and the total numbers of CFU in the lungs were measured. A noninfected group of mice served as controls for efficiency of the infection (Fig. 1).

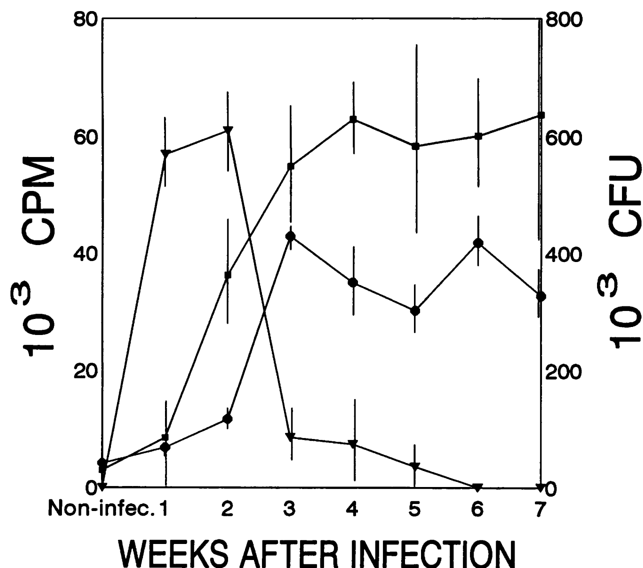

FIG. 1. Proliferative responses of lymphocytes isolated from the TBL (■) and SPL (●) of mice infected with live, virulent *B. pertussis* bacteria and stimulated in vitro with killed WB (12.5×10^7 bacteria per ml). The courses of the infections were measured by counting CFU (▼) in lung smears from the mice. The proliferative responses and CFU were measured 1 to 7 weeks following the infection. The responses of noninfected mice (Non-infec.) are indicated to the left. The points represent means of results for 3 to 5 mice. Thin bars show standard deviations. The experiments were performed twice, with similar results.

The total numbers of CFU in the lungs of the infected mice reached a maximum 2 weeks following the infection and then declined. The bacteria were cleared from the lungs 6 weeks following the infection. The numbers of CFU recovered from the lungs of the infected mice were significantly higher than the numbers of CFU recovered from the group of noninfected mice ($P < 0.005$) at weeks 1, 2, 3, 4, and 5 after the infection. The bacterial load in the lungs of a group of mice sacrificed 1 h after the infection was 21,400 ± 2,104 CFU ($n = 5$). The bacterial load in the lungs of the mice 1 to 2 weeks after the infection was 25 times as high, indicating a vigorous proliferation of the bacteria during the first 2 weeks of infection.

Proliferative responses of lymphocytes from TBL and SPL. The in vitro proliferative responses of TBL and SPL isolated from infected mice and from noninfected controls to stimulation with intact *B. pertussis* organisms were measured (Fig. 1). The proliferative responses of SPL and TBL increased from week 1 to weeks 3 and 4 after the infection, respectively, to a plateau level. The proliferative responses of TBL from infected mice were significantly higher than the responses of TBL from noninfected mice at 2 to 7 weeks after the infection ($P < 0.03$). Likewise, the proliferative responses of SPL from infected mice were significantly higher than the responses of SPL from noninfected mice at 1 to 7 weeks after the infection ($P < 0.02$). The responses of TBL against *B. pertussis* were significantly higher than the response of SPL at 2 to 7 weeks after the infection ($P < 0.02$).

Measurement of IFN-γ and TNF. The IFN-γ and TNF levels in culture supernatants of TBL and SPL stimulated with intact *B. pertussis* bacteria were assessed (Fig. 2). The TBL and SPL were isolated from the infected mice as reported above for the proliferation tests. The IFN-γ levels in the culture supernatants of both TBL and SPL peaked 3 to 4 weeks after the infection ($P < 0.05$). The IFN-γ levels in the SPL supernatants were higher than in the TBL supernatants from week 3 after the infection. This was statistically significant for weeks 3, 4, and 6 ($P < 0.05$).

The TNF level peaked 2 weeks after the infection for both TBL and SPL ($P < 0.05$) and thereafter returned to the TNF level of lymphocytes isolated from the noninfected group. Generally, the TNF level in the SPL culture supernatants was higher than that in the TBL supernatants; this was statistically significant at all time points after the infection ($P < 0.02$).

Antibody response of the infected mice. The serum antibody responses against a range of *B. pertussis* antigens in sera from the infected mice were measured. Initially, the antibody responses of individual mice against intact *B. pertussis* were measured (results not shown). Because differences in the titers between individual mice were small within the groups, the sera were pooled.

The pooled sera were then tested against WB and a range of purified pertussis antigens by ELISA (Fig. 3). A low antibody response against WB appeared 3 weeks after the infection. This response remained low until weeks 6 and 7, when the response increased sharply. Of the antibody responses to the individual purified *B. pertussis* antigens, only the response to FHA reached detectable levels, but this response remained weak during the study period. Seven weeks after the infection, the serum antibody response against intact *B. pertussis* organisms was still several log units below the response that can be induced by parenteral immunization of mice with whole-cell pertussis vaccine (unpublished results).

Proliferative responses to purified *B. pertussis* antigens. The proliferative responses and the IFN-γ levels were maximal in the TBL and SPL cultures 3 weeks after the infection. On the basis of these results, a new group of mice was aerosol infected, and the proliferative responses as well as the IFN-γ and TNF production levels to purified *B. pertussis* antigens were measured 3 weeks after the infection.

When TBL from the infected mice were stimulated with WB, native PT, PT detoxified with chemical (dPT-F and dPT-H) or recombinant (dPT-r) techniques, FHA, the 69-kDa OMP, Agg 2, and Agg 3, as well as a hitherto uncharacterized *B. pertussis* antigen with a molecular mass of 80 kDa isolated in our laboratory, all stimulated vigorous proliferative responses (Fig. 4). Statistical analysis of the proliferative responses of the TBL and SPL of the infected groups compared with the proliferative responses of the noninfected groups was based on index values (response of antigen-stimulated cultures/response of unstimulated cultures), because the proliferative responses in the unstimulated cultures of the infected mice were higher than the corresponding proliferation responses of the noninfected group. The results of the analysis showed that the proliferative responses to WB, dPT-H, FHA, the 69-kDa OMP, Agg 2, and Agg 3, as well as to the 80-kDa antigen, of the TBL from the infected group were significantly higher than the responses of the TBL from the noninfected group ($P < 0.01$). For the SPL, the proliferative responses to WB, native PT, dPT-F, dPT-H, FHA, the 69-kDa OMP, Agg 2, and Agg 3, as well as to the 80-kDa antigen, were significantly higher ($P < 0.005$) in the infected group than in the noninfected group.

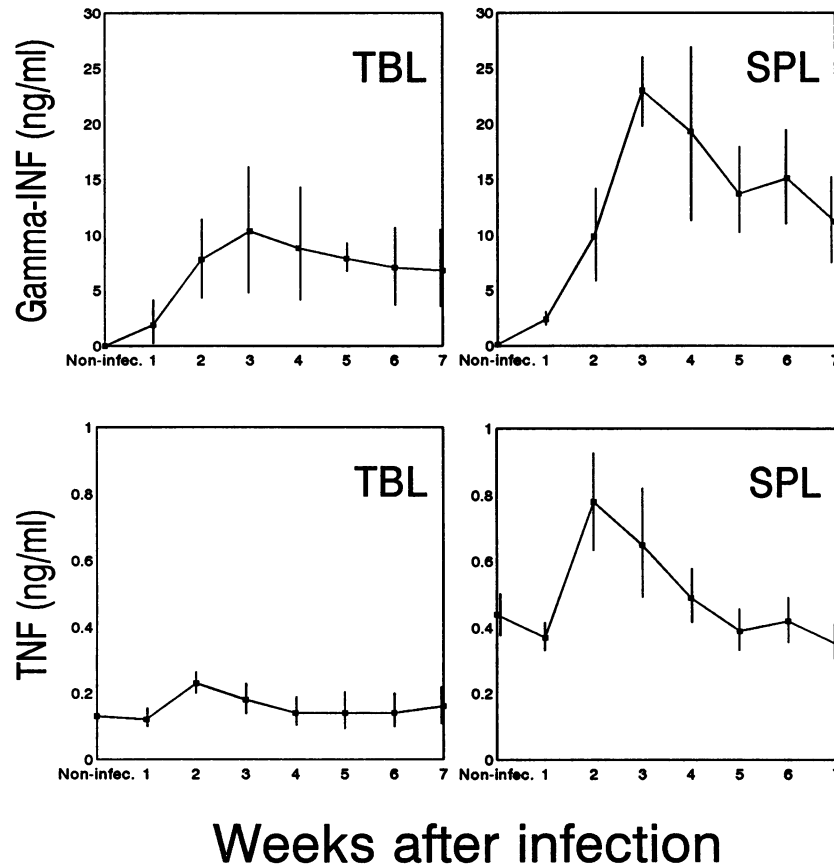

FIG. 2. In vitro IFN-γ and TNF levels in culture supernatants of TBL and SPL isolated from *B. pertussis*-infected mice and stimulated in vitro with killed WB (12.5×10^7 bacteria per ml). The responses of noninfected mice (Non-infec.) are indicated to the left. Points represent means of lymphocyte cultures from 1 to 5 individual mice. Thin bars show standard deviations.

The proliferative responses of the SPL thus showed a pattern of reaction to the purified antigens similar to that of the TBL. The results of a comparison of the response of the TBL to the response of the SPL showed that the response to WB, dPT-H, FHA, the 69-kDa OMP, Agg 2, and Agg 3, as well as to the 80-kDa antigen, were significantly higher ($P < 0.05$) for the TBL than for the SPL. This confirmed the results obtained in the study of the infection kinetics.

IFN-γ and TNF response to purified *B. pertussis* antigens. The IFN-γ and TNF levels in supernatants of TBL and SPL cultures stimulated with purified *B. pertussis* antigens between groups of noninfected mice and mice infected for 3 weeks with *B. pertussis* were compared.

The TBL supernatants from the infected group had a statistically significantly higher ($P < 0.005$) level of IFN-γ than the TBL from the noninfected group for PHA, WB, native PT, FHA, Agg 2, Agg 3, and the 80-kDa antigen (Table 1). For the SPL supernatants, the IFN-γ levels were significantly higher ($P < 0.03$) in the infected group for PHA, WB, native PT, dPT-r, FHA, Agg 3, and the 80-kDa antigen. When the levels of IFN-γ in the culture supernatants of TBL and SPL from the infected group were compared, the results showed that the TBL had a significantly higher ($P < 0.03$) IFN-γ level than the SPL for FHA, Agg 2, Agg 3, and the 80-kDa antigen.

The TBL supernatants from the infected group had a significantly higher ($P < 0.03$) level of TNF than the TBL from the noninfected group for PHA, WB, PT, dPT-r, FHA, Agg 2, Agg 3, and the 80-kDa antigen (Table 2). Because the supernatants from the unstimulated SPL contained detectable levels of TNF, these values were subtracted from the levels in the supernatant from the SPL cultures stimulated with the purified antigens prior to the statistical analysis. The results showed that the SPL from the infected group had a significantly higher level of TNF than the SPL supernatants from the noninfected group for PHA, WB, PT, and dPT-r ($P < 0.01$).

Identification of lymphocyte subsets. In order to identify which subpopulations of T cells proliferated in response to the purified antigens, the in vitro proliferative responses of $CD4^+$ and $CD8^+$ lymphocytes were blocked with anti-$CD4^+$ and anti-$CD8^+$ MAbs, respectively (Fig. 5). The proliferative responses to all antigens were completely or partially blocked by addition of the anti-$CD4^+$ MAb, indicating that the responses to these antigens were at least in part mediated by T-helper lymphocytes. The residual proliferation caused by WB, Agg 2, and Agg 3 can be explained by trace amounts of lipopolysaccharide in these preparations, stimulating minor, unspecific B-cell proliferation. The effect of the addition of anti-$CD4^+$ MAbs was statistically significant for WB, dPT-H, FHA, Agg 2, Agg 3, and the 80-kDa antigen ($P < 0.05$) for the TBL and for WB, dPT-H, FHA, the 69-kDa OMP, Agg 2, Agg 3, and the 80-kDa antigen ($P < 0.05$) for the SPL.

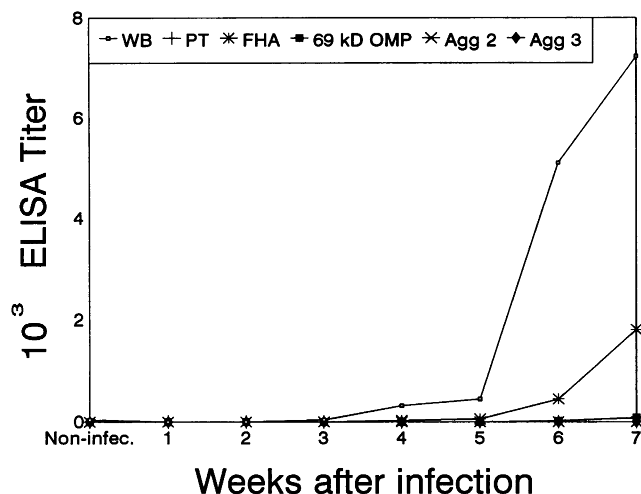

FIG. 3. Serum antibody responses of infected mice against killed WB and purified *B. pertussis* antigens. The responses of noninfected mice (Non-infec.) are indicated to the left. The antibody titers were determined in serum pools from five mice. Antisera or MAbs against the individual antigens were used as standards in the ELISA.

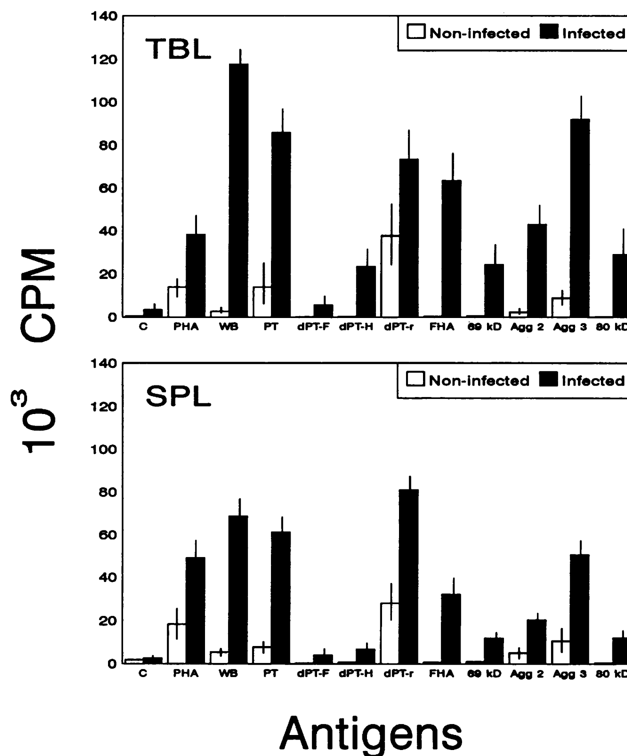

FIG. 4. Proliferative responses of TBL and SPL from infected and noninfected mice to purified *B. pertussis* antigens. The lymphocytes from the infected mice were isolated 3 weeks after the infection. The concentrations of the antigens were based on previous titrations and were as follows: PHA, 1 μg/ml; WB, 12.5 × 10^7 bacteria per ml; PT, 0.2 μg/ml; dPT-r, 0.1 μg/ml; Agg 2 and Agg 3, 2.5 μg/ml; 80-kDa antigen, 1 μg/ml; dPT-F, dPT-H, FHA, and the 69-kDa OMP, 10 μg/ml. Bars represent means of four to six pools of lymphocytes (1 to 15 mice per pool). Thin bars show standard deviations. C, control (cultures without antigen).

DISCUSSION

The results of the present study show that from 1 to 3 weeks following aerosol infection of mice with live, virulent *B. pertussis* bacteria, a strong T-cell-mediated reactivity to intact *B. pertussis* bacteria is developed in the TBL and the SPL of the mice. The responses of the TBL are generally higher than the responses of the SPL. The bacterial load in the lungs of the mice is maximal 2 weeks after the infection but thereafter rapidly declines, as reported by others (28, 31), concurrently with the increase to the maximum level of CMI in the TBL and SPL. As likewise observed with human *B. pertussis* infection, measurable levels of *B. pertussis*-specific serum antibodies emerge only when the infection is almost cleared (21). This points to a crucial role of CMI in the clearance of *B. pertussis* infection.

IFN-γ and TNF are potent activators of various cell types operating in CMI, e.g., macrophages and T-cells, especially in the context of intracellular parasites (7). The present study shows that a specific in vitro production of IFN-γ and TNF accompanies the time course of the proliferative responses of the TBL and SPL. IFN-γ is secreted solely by T-helper 1 cells (18), which are of importance in the activation of macrophages and other cell types with microbicidal activities (19). Therefore, these results give further support for the idea that CMI plays a decisive role in the clearance of *B. pertussis* infection of mice.

In order to evaluate the involvement of defined antigens in the response to *B. pertussis*, the proliferative responses as well as the IFN-γ and TNF production levels were measured 3 weeks after the infection. Specific proliferative responses, mediated by T cells of the helper phenotype, were found against PT, FHA, the 69-kDa OMP, Agg 2, and Agg 3, antigens which are all considered candidates for inclusion in future acellular pertussis vaccines (29). The response of the TBL was generally higher than the response of the SPL, thus confirming the results of the study of the time course of the proliferative responses. CMI against FHA, the 69-kDa OMP, Agg 2, and Agg 3 has been demonstrated for humans following parenteral immunization with whole-cell pertussis vaccine and for human pertussis convalescents (6, 11, 24, 38, 39). In mice, CMI against FHA, the 69-kDa OMP, Agg 2, and Agg 3 has been demonstrated following parenteral immunization with whole-cell pertussis vaccine (9, 10, 22). The role of PT in CMI against *B. pertussis* is still controversial. Some studies have shown CMI against PT following infection with *B. pertussis* or parenteral immunization with whole-cell pertussis vaccine (6, 9, 10, 11, 20, 38), while other studies have failed to do so (2, 22). It has been suggested that the lack of response to PT is due to destruction of T-cell epitopes on PT during the detoxification process (22). Moreover, PT is a potent T-cell mitogen (16) and the in vitro mitogenic activity of PT may mask antigen-specific proliferative responses (38). Therefore, in the present study, we chose to evaluate the responses not only to native PT but also to PT detoxified by different methods. dPT-r, detoxified by recombinant technology, is, like native PT, a potent mitogen for murine T cells (22); accordingly, no specific proliferative responses to this antigen could be detected. The PT preparations detoxified by chemical methods, dPT-F and dPT-H, are known to be without in vitro mitogenic activities (22). The results of in vitro stimulation with dPT-H show that T cells with specificity for PT are induced during aerosol infection of mice.

In a recent study, we have shown that *B. pertussis* antigens other than the ones currently under consideration for inclusion in acellular pertussis vaccines might be in-

TABLE 1. In vitro IFN-γ production in response to purified antigens[a]

Antigen	Concn (μg/ml)	IFN-γ production (ng/ml)			
		Noninfected TBL lymphocytes (n)[b]	Infected TBL lymphocytes (n)	Noninfected SPL lymphocytes (n)	Infected SPL lymphocytes (n)
Control		0 (8)	0 (6)	0 (8)	0 (6)
PHA	1	0.4 ± 0.2 (8)	4.2 ± 1.5 (6)	4.3 ± 1.4 (8)	10.2 ± 4.8 (6)
WB	—[c]	0.2 ± 0.3 (4)	26.7 ± 6.1 (6)	1.8 ± 0.2 (4)	31.5 ± 9.5 (6)
PT	0.2	0 (4)	1.4 ± 0.08 (6)	0.1 ± 0.1 (4)	1.5 ± 0.4 (6)
dPT-F	10	0 (4)	0 (6)	0 (4)	0 (6)
dPT-H	10	0 (4)	0.2 ± 0.3 (6)	0 (4)	0 (6)
dPT-r	0.1	0 (4)	1.3 ± 0.6 (6)	0.2 ± 0.2 (4)	1.9 ± 1.0 (6)
FHA	10	0 (4)	1.9 ± 1.1 (6)	0 (4)	0.4 ± 0.3 (6)
69 kDa	10	0 (4)	0.2 ± 0.3 (6)	0 (4)	0.1 ± 0.2 (6)
Agg 2	2.5	0 (4)	2.2 ± 1.4 (6)	0 (4)	0.4 ± 0.5 (6)
Agg 3	2.5	0.1 ± 0.1 (4)	5.4 ± 3.5 (6)	0 (4)	1.3 ± 1.2 (6)
80 kDa	1	0.1 ± 0.2 (4)	1.0 ± 0.6 (6)	0 (4)	0.4 ± 0.3 (6)

[a] Lymphocytes were cultured for 72 h. The concentrations of IFN-γ in the culture supernatants were determined by an ELISA (see Materials and Methods).
[b] n, number of pools of lymphocytes (1 to 15 mice per pool).
[c] —, 12.5 × 10^7 bacteria per ml.

volved in CMI against *B. pertussis* following parenteral immunization of mice with whole-cell pertussis vaccine (22). Therefore, we included a novel purified *B. pertussis* antigen with a molecular mass of 80 kDa in the present study. A proliferative response mediated by T cells of the helper phenotype against this antigen was likewise found.

The results of the IFN-γ and TNF secretions in response to the purified antigens show that especially the responses to FHA and to Agg 2 and Agg 3, which are important in the adherence of the *B. pertussis* organism to the ciliated epithelium in the respiratory tract (15, 27), are higher for the TBL than for the SPL. This may point to a special role for FHA, Agg 2, and Agg 3 in the regional CMI of the respiratory tract.

The IFN-γ and TNF responses to native PT and dPT-r were also found to be higher in the infected mice than in the noninfected mice. However, these results should be considered with caution, since the PHA-stimulated cultures also had higher levels of IFN-γ and TNF for the infected group. Because no significant differences between the responses of infected and noninfected mice to the nonmitogenic dPT-F and dPT-H were found, it must be concluded that no antigen-specific IFN-γ and TNF production was found in response to PT.

The specific IFN-γ and TNF responses to the 69-kDa OMP were generally weak or absent. The specific proliferative responses to the 69-kDa OMP proved that the lymphocytes reacted to this antigen. This may point to a minor importance for the 69-kDa OMP with respect to induction of these two cytokines.

In conclusion, the present study is the first to show that aerosol infection of mice with virulent *B. pertussis* organisms induces CMI in the regional immune system of the airway, represented by the TBL, as well as in the systemic immune system, represented by the SPL. The development of CMI is paralleled by the clearance of the infection from the lungs. A *B. pertussis*-specific antibody response in the sera of infected mice is developed only when the infection is almost cleared. In addition, the involvement of several purified *B. pertussis* antigens in CMI has been demonstrated.

Future studies will be focused on regional cytokine and antibody secretion in the lymph nodes draining the airways of *B. pertussis*-infected mice.

TABLE 2. In vitro TNF production in response to purified antigens[a]

Antigen	Concn (μg/ml)	TNF production (ng/ml)			
		Noninfected TBL lymphocytes (n)[b]	Infected TBL lymphocytes (n)	Noninfected SPL lymphocytes (n)	Infected SPL lymphocytes (n)
Control		0 (8)	0 (6)	0.03 ± 0.03 (8)	0.11 ± 0.04 (6)
PHA	1	0.05 ± 0.03 (8)	0.20 ± 0.06 (6)	0.13 ± 0.04 (8)	0.33 ± 0.10 (6)
WB	—[c]	0.03 ± 0.03 (4)	0.33 ± 0.08 (6)	0.51 ± 0.07 (4)	0.90 ± 0.17 (6)
PT	0.2	0 (4)	0.08 ± 0.05 (6)	0.01 ± 0.03 (4)	0.15 ± 0.05 (6)
dPT-F	10	0 (4)	0 (6)	0 (4)	0.05 ± 0.06 (6)
dPT-H	10	0 (4)	0.02 ± 0.05 (6)	0.06 ± 0.02 (4)	0.12 ± 0.10 (6)
dPT-r	0.1	0.01 ± 0.03 (4)	0.06 ± 0.04 (6)	0.03 ± 0.03 (4)	0.17 ± 0.04 (6)
FHA	10	0 (4)	0.09 ± 0.02 (6)	0 (4)	0.13 ± 0.04 (6)
69-kDa	10	0 (4)	0 (6)	0.05 ± 0.04 (4)	0.09 ± 0.05 (6)
Agg 2	2.5	0.01 ± 0.03 (4)	0.09 ± 0.05 (6)	0.17 ± 0.03 (4)	0.25 ± 0.06 (6)
Agg 3	2.5	0 (4)	0.12 ± 0.02 (6)	0.15 ± 0.03 (4)	0.39 ± 0.35 (6)
80-kDa	1	0 (4)	0.07 ± 0.04 (6)	0.05 ± 0.04 (4)	0.15 ± 0.03 (6)

[a] Lymphocytes were cultured for 72 h. The concentrations of TNF in the culture supernatants were determined by ELISA (see Materials and Methods).
[b] n, number of pools of lymphocytes (1 to 15 mice per pool).
[c] —, 12.5 × 10^7 bacteria per ml.

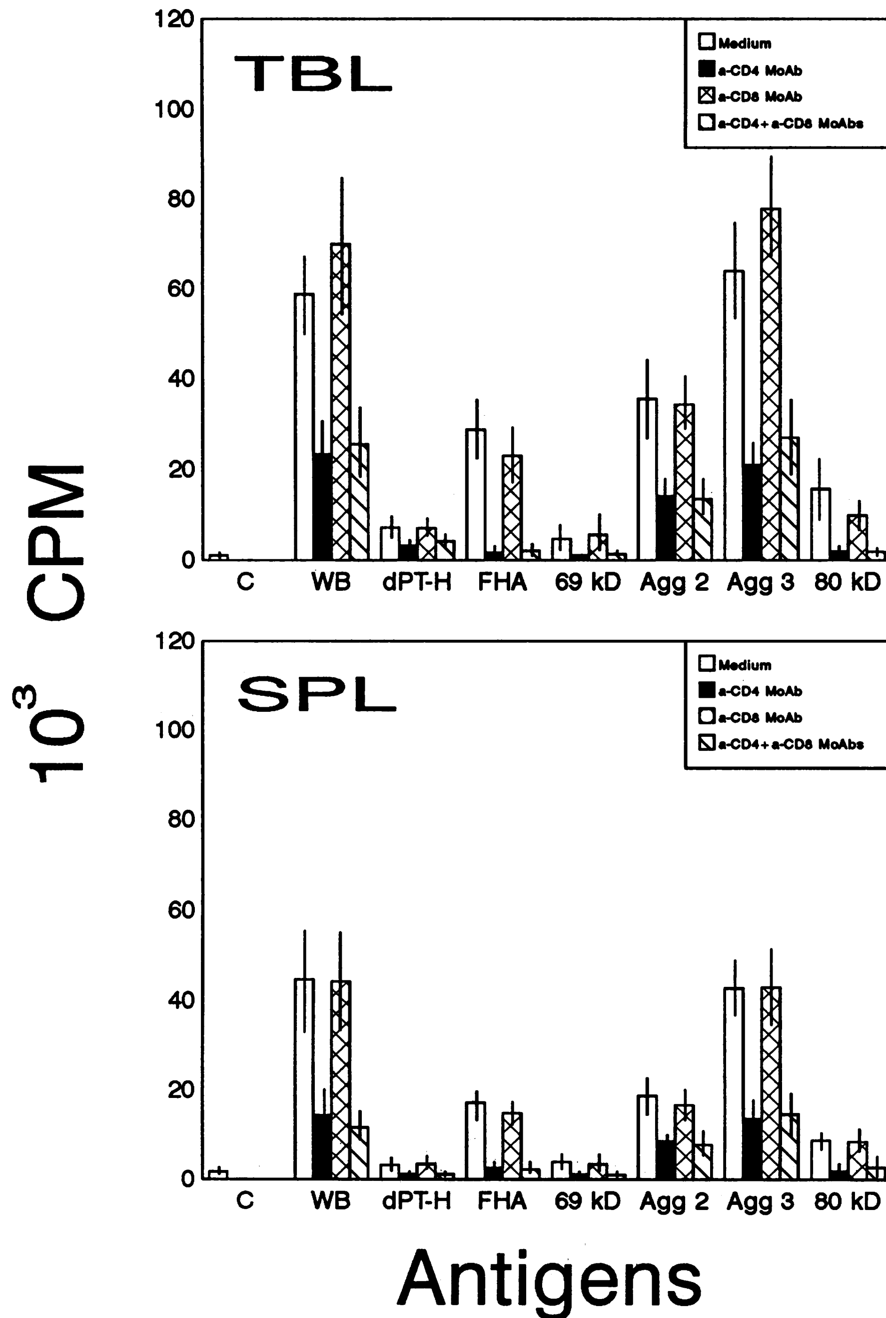

FIG. 5. Proliferative responses of TBL and SPL from infected mice to purified *B. pertussis* antigens and blocked by MAbs against the CD4 and CD8 molecule. Lymphocytes were isolated 3 weeks after the infection. Bars represent means results from six mice. Thin bars show standard deviations. For in vitro antigen concentrations, see the legend to Fig. 4. C, control (cultures without antigen).

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