INFECTION AND IMMUNITY, Apr. 1984, p. 1–6
0019-9567/84/040001-06$02.00/0

Vol. 44, No. 1

# Proliferative Response of Immune Mouse T-Lymphocytes to the Lymphocytosis-Promoting Factor of *Bordetella pertussis*

FALK FISH,[1,2] JAMES L. COWELL,[1] AND CHARLES R. MANCLARK[1]*

*Division of Bacterial Products, National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Maryland 20205,[1] and Department of Microbiology, Tel Aviv University, Tel Aviv, Israel[2]*

Received 22 September 1983/Accepted 15 December 1983

Immunization of mice with a whole-cell pertussis vaccine or with the purified, detoxified lymphocytosis-promoting factor (LPF) of *Bordetella pertussis* resulted in an increased in vitro proliferative response to LPF in immune lymph node cells. The proliferative response was detected above the nonspecific mitogenic activity of LPF. That the proliferative response of the immune lymph node cells was a demonstration of a specific cell-mediated immunity to LPF was supported by the following: (i) the specificity of the response to the immunizing antigen; (ii) the ability of chemically modified, nonmitogenic LPF to induce proliferation in immune lymph node cells; and (iii) a dependence on T-cells for the demonstration of the proliferative response of immune cells to LPF. Immunization of mice with protective doses of detoxified LPF resulted in serum antibody and cell-mediated responses to LPF. Immunization of mice with protective doses of whole-cell pertussis vaccine resulted in a cell-mediated response but not a detectable antibody response to LPF. The LPF of *B. pertussis* is believed to play an important role in pathogenesis and immunity in pertussis, and the demonstration of a cell-mediated immune response to LPF suggests a possible role for cell-mediated immunity to LPF in protection from pertussis disease.

The proper use of a vaccine consisting of whole *Bordetella pertussis* cells effectively protects against pertussis. The protection of mice, conferred by the whole-cell vaccine, against intracerebral challenge with live *B. pertussis* has been shown to correlate with the clinical efficacy of pertussis vaccine for humans (12, 13, 23). The lymphocytosis-promoting factor (LPF; also known as histamine-sensitizing factor, islet-activating protein, pertussigen, pertussis toxin) is a protein toxin produced by *B. pertussis* which has many biological activities and effects, including lethality, promotion of lymphocytosis, histamine sensitization, adjuvant effects, and potentiation of insulin secretion (15). The toxin is thought to be responsible for many symptoms of pertussis (18), and a toxoid prepared by glutaraldehyde treatment of purified LPF has been shown to protect mice against the standard intracerebral challenge with *B. pertussis* (5, 16). Due to the apparent importance of LPF in disease and protection from disease, the immune responses to LPF, elicited by the whole-cell vaccine and purified LPF, were analyzed.

The serum antibody response was measured by enzyme-linked immunosorbent assay (ELISA). Of the many methods for demonstrating and assaying cell-mediated immune responses, the specific in vitro proliferation of lymphoid cells (17) was chosen. A specific cell-mediated proliferative response to LPF was partially masked by the inherent T-cell mitogenic activity of LPF (10, 14). It was possible, however, to separate the mitogenic response from antigen-induced proliferation and to demonstrate that whole-cell vaccine elicits an immunological, specific T-cell-mediated, proliferative lymphoid cell response to LPF as an immunogen. Since immunization of mice with pertussis vaccine or detoxified LPF protects them from challenge with *B. pertussis*, a role for cell-mediated immunity in protection from pertussis is suggested.

* Corresponding author.

## MATERIALS AND METHODS

**Mice.** C57BL/6 female mice 8 to 12 weeks old were obtained from Microbiological Associates, Walkersville, Md., or from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

**Proteins.** LPF was purified from supernatants of *B. pertussis* Tohama grown in stationary cultures or *B. pertussis* 165 grown in shake cultures by the method of Cowell et al. (5) and Sato et al. (20). LPF also was prepared by the method of Sekura et al. (22a). LPF obtained by both methods was indistinguishable by its in vitro immunogenic and mitogenic activities. The filamentous hemagglutinin (FHA) of *B. pertussis* was purified from supernatants of strain Tohama stationary cultures (5, 20). Both antigens contained less than 1% lipid and carbohydrate as measured by colorimetric assay (20).

**Immunizations.** Mice were injected intraperitoneally on days 0 and 14 with 4 opacity units of pertussis vaccine lot no. 7b or 5 μg of LPF or FHA without aluminum adjuvant. Pertussis vaccine lot no. 7b is a whole-cell preparation of killed *B. pertussis* 27 preserved with 0.01% thimerosal and freeze-dried. The LPF used for immunization was detoxified with glutaraldehyde by the method of Munoz et al. (16). These doses are protective in the mouse intracerebral challenge assay for pertussis vaccine and LPF but not for FHA (data not shown). Mice were bled, and lymphoid cells were harvested 2 weeks after the last injection. Control treatment mice received saline injections.

**Assays for immune responses.** Serum antibody levels were measured by a modification of the microELISA method of Engvall and Perlman (6). Briefly, 0.05 ml of LPF solution (2 μg/ml) or pertussis vaccine lot no. 7b (20 opacity units per ml), both in 0.1 M $NaHCO_3$ (pH 9.0) buffer, was introduced into the wells of Immulon microtitration plates (Dynatech Laboratories Inc., Alexandria, Va.). After 1 h of incubation at room temperature, the plates were washed with 0.1% Brij 35 in phosphate-buffered saline (PBS) (PBS-Brij), employing a Multiwash plate-washing apparatus (Flow Laboratories,

Inc., McLean, Va.). Heat-inactivated sera (30 min, 56°C), diluted 1:10 in PBS-Brij, were then added to antigen-coated or antigen-free wells and incubated for 3 h at room temperature. After another wash with PBS-Brij, the plates were incubated at room temperature with a conjugate of alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) and affinity-purified goat anti-mouse Fab antiserum (Cappel Laboratories, West Chester, Pa.).

A 1-mg/ml solution of *p*-nitrophenylphosphate (Calbiochem-Behring, La Jolla, Calif.) in 1 M Tris-hydrochloride buffer (pH 9.0) with 0.3 mM $MgCl_2$ was introduced into all wells, and the developing color was measured in a Dynatech AutoELISA reader at 405 nm. The optical density values were corrected for 1-h color development.

In vitro cell-mediated immunity was assessed by the antigen-specific proliferative response of immune lymphoid cells as compared with the response of cells derived from control animals. The methods for harvesting spleen and mesenteric lymph node cells, T-cell depletion, culturing, and measuring cell proliferation were described by Fish and Ziff (7), based in part on the method of Alkan (1) for the demonstration of T-cell proliferative responses to antigen. Lipopolysaccharide W of *Escherichia coli* O55:B5 (LPS; Difco Laboratories, Detroit, Mich.) and concanavalin A (ConA; Miles-Yeda, Ltd., Rehovot, Israel), B- and T-cell mitogens, respectively, were used in the culture systems to assess the efficacy of the depletion procedures and the viability and responsiveness of pertinent cell populations. Values obtained from scintillation counts were expressed as the differences in counts of tritiated thymidine incorporation per minute between antigen-treated microcultures and control cultures (delta cpm). Stimulation index (SI) values were calculated by the following equation: SI = delta cpm of immune cells/delta cpm of normal cells.

**Chemical modification of LPF.** Based on the procedures of Lönnroth and Holmgren (12), LPF was dialyzed against PBS, and the protein concentration was estimated based on absorption at 280 and 260 nm (11) and was adjusted to 0.1 mg/ml. Trinitrobenzenesulfonic acid (TNBS; Sigma) was added to a final concentration of 1 mM in the presence of 0.1 M sodium carbonate. After 3 h of incubation at room temperature in the dark, the incubation mixture was dialyzed against PBS to remove unreacted TNBS. For iodination, potassium iodide was added to LPF to a final concentration of 0.1 M. Chloramine-T (Eastman Kodak Co., Rochester, N.Y.) was then added to a final concentration of 0.25 mg/ml, and 1 min thereafter it was neutralized with sodium metabisulfite (1-mg/ml final concentration). The concentrations of TNBS, potassium iodide, and chloramine-T were the minimum required to effect complete detoxification.

## RESULTS

**In vitro proliferative response to LPF in normal and vaccinated mice.** To demonstrate the existence of a specific proliferative response to LPF in mice injected with whole-cell pertussis vaccine, spleen and lymph node cells from normal (saline-injected) and vaccinated mice were cultured for 3 days with various concentrations of LPF. In preliminary experiments different durations of incubation were tried; 3 days was found to give the maximal proliferative response in normal and immune animals. The same lymphoid cell populations were also cultured with the optimal amounts of the mitogens LPS and ConA. The radioactivity incorporation values are presented in Fig. 1.

No evidence for a specific proliferative response to LPF was found for spleen cells at concentrations of LPF ranging from 0.1 to 5.0 μg/ml. In all cases, spleen cells from normal animals responded to LPF better than did cells from immune animals. It should be noted, however, that the responses of immune spleen cells to the nonspecific mitogenic stimuli of LPS and ConA were also suppressed, compared with the responses of their normal counterparts. As was determined by cell-mixing experiments, the mechanisms for the decreased reactivity of the immune spleen cells do not involve suppressor cells.

Mesenteric lymph node cells from vaccinated mice demonstrated a specific proliferative response. This response was accentuated at a nonmitogenic LPF concentration of 0.5 μg/ml. At this concentration of LPF, immune lymph node cells proliferated, whereas normal lymph node cells did not show any measurable proliferative response over background levels. A difference in the proliferative response between normal and immune lymph node cells was maintained at higher concentrations of LPF, when the mitogenic activity of LPF began to show its effect. At the highest concentration of LPF used, 5 μg/ml, the difference between normal and immune cells was reduced, since the mitogenic activity of LPF was at its optimum (2.5 to 5 μg/ml). In addition, the control cultures with LPS and ConA showed that both cell populations were equally responsive for B- and T-cell mitogens, suggesting that the difference observed in the response to LPF represented a specific cell-mediated proliferative response to LPF as an immunogen.

**Proliferative response to modified LPF.** One of the difficulties in demonstrating the specific proliferative response to LPF is the inherent mitogenic activity of the protein, which results in a high "background" response in normal cell populations. This difficulty could be overcome if the mitogenic activity of LPF was eliminated without affecting to a large extent the antigenic or immunogenic structure of the molecule.

After various protein modification reagents, each exhibiting a certain degree of specificity to certain amino acid residues, were screened, TNBS was chosen for further studies. TNBS is a well-known ε-amine modifying agent, and the modification reaction is carried out under mild conditions. Modification did not alter the antigenic integrity of LPF, as determined by ELISA (6), but TNBS was effective in eliminating the mitogenic activity of LPF (Fig. 2). The two upper curves in Figure 2 represent the in vitro proliferative response to a different LPF preparation than was used in the experiment shown in Fig. 1. Lymph node cells derived from normal mice and mice immunized with pertussis vaccine were cultured with various concentrations of native or TNBS-modified LPF (Fig. 2). The proliferative response of immune cells was stronger than that of normal cells, and the difference was maintained at the suboptimal mitogenic concentration of native LPF. Lymph node cells obtained from normal animals, however, did not respond to modified LPF. Immune cells did proliferate when cultured with the otherwise nonmitogenic modified LPF, thus providing further evidence that the heightened proliferative response of immune lymph node cells is a result of both mitogenic and specific antigenic stimulations. It did appear, however, that TNBS modification of LPF also reduced the specific proliferative activity of LPF.

**Role of T-cells in the proliferative response to LPF.** The T-cell compartment of the immune system is responsible for generating cell-mediated responses. Therefore, the role of T-cells in the in vivo proliferative response as an indication of cell-mediated immunity was tested.

Normal and immune lymph node cells were treated with

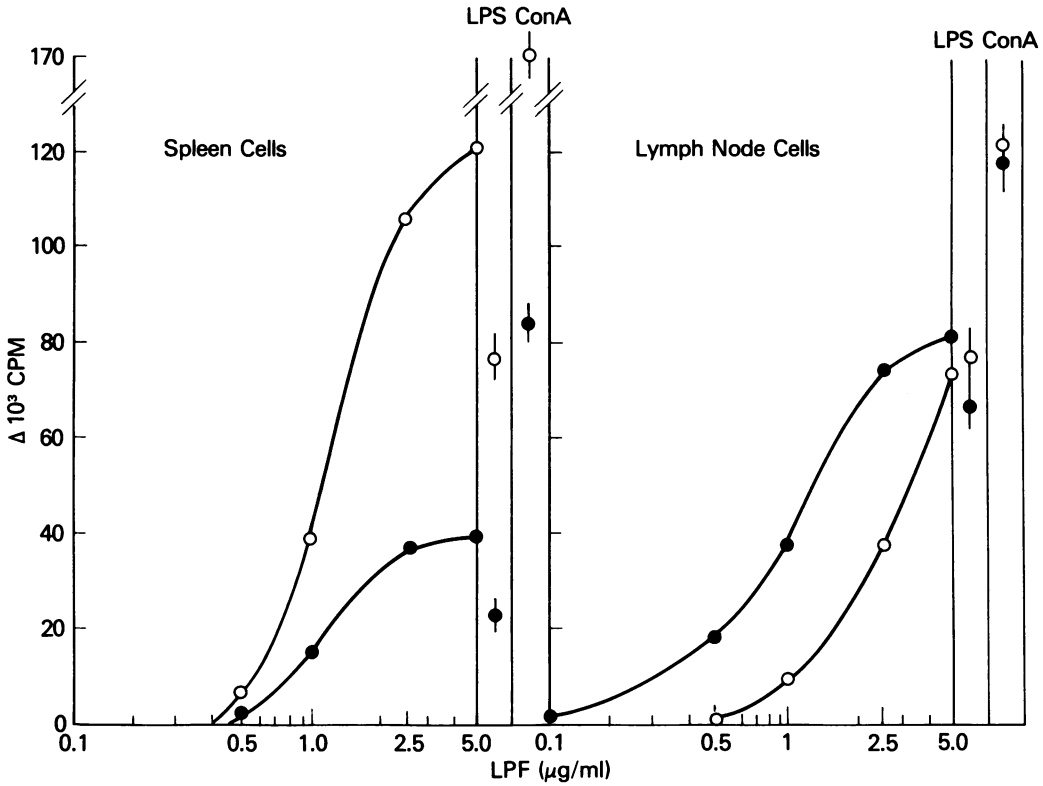


FIG. 1. In vitro proliferative response to LPF in normal (saline-treated) and vaccinated mice (six animals in each group). Lymphoid cells were harvested after two injections of saline or pertussis vaccine lot no. 7b. The responses to LPS and ConA are used as indicators for the in vitro responsiveness of the cells. The values presented are the means of six replicate cultures. Symbols: ○, normal cells; ●, immune cells.

anti-Thy-1.2 hybridoma antibody and complement to eliminate T-cells. The untreated cell populations and their T-cell-depleted counterparts were cultured with LPS, ConA, and various concentrations of LPF as described above. The removal of T-cells eliminated the mitogenic response from both normal and immune cells in addition to eliminating the specific response of immune lymph node cells to the 0.25-μg/ml submitogenic concentration of LPF (Table 1). Based on this result and the fact that the T-cell-depleted population still had reactive B-cells (they responded to LPS), it can be concluded that the specific proliferative response to LPF in vaccinated mice is T-cell dependent.

**Specificity of the in vitro proliferative response.** To support the concept of specific cell-mediated immunity to LPF, the specificity of the response to the immunizing antigen was determined. Mice were immunized with either pertussis vaccine, LPF, or FHA. Lymph node cells obtained from each of the immunization groups were cultured with two representative concentrations of LPF or FHA, and the proliferation was assayed 3 days later, as described above. Lymph node cells derived from animals immunized with LPF or whole bacteria demonstrated an accentuated proliferative response to LPF compared with normal cells (Fig. 3). Lymphoid cells of FHA-immune animals did not respond to LPF better than normal cells. Conversely, LPF-immune and normal lymph node cells responded to FHA in an identical fashion, whereas FHA- or pertussis vaccine-immune lymph node cells demonstrated an increased proliferation in the presence of FHA. FHA is not a mitogen; therefore, the background response to this protein is low compared with the response to LPF.

**In vitro proliferative response after immunization with purified LPF.** Since LPF is only one of many components of whole-cell pertussis vaccine, it was of interest to determine whether the purified antigen would induce specific cell-mediated immunity. Mice were immunized with iodine-detoxified LPF, and their lymph node cells were cultured with LPF as described above. It appeared that immunization with LPF alone was sufficient to induce an increased in vitro proliferative response to LPF in immune lymph node cells (Fig. 4).

**Serum antibody and cell-mediated responses to LPF.** Table 2 summarizes the mean immune response values of 50 animals, each of which received two injections of either pertussis vaccine or glutaraldehyde-detoxified LPF. The serum antibody response was assessed by ELISA, which was developed with a polyvalent anti-immunoglobulin antibody. The cell-mediated response to LPF was assessed by the in vitro proliferation assay, which has been shown to be a true indication of a T-cell-mediated immune response. The most important observation in Table 2 is the absence of an ELISA-detectable serological response to LPF after immunization with an otherwise protective dose of a whole-cell vaccine. That the animals were able to generate such a response to LPF was exemplified by the group of mice which was immunized with purified glutaraldehyde-detoxified LPF. Both groups of mice demonstrated a cell-mediated response to LPF of comparable magnitude.

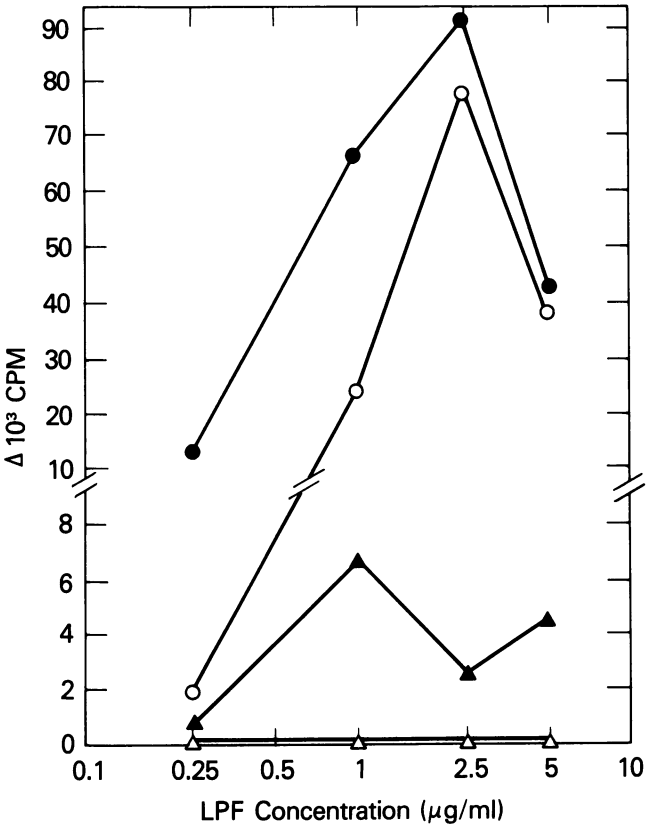


FIG. 2. In vitro proliferative response to native and modified LPF. Lymph node cells were harvested from saline-injected (normal) or from pertussis vaccine-injected mice (six animals in each group). LPF was modified by treatment with TNBS. The values presented are the means of six replicate cultures. Symbols: ○, normal cells response to native LPF; ●, immune cells response to native LPF; △, normal cells response to modified LPF; ▲, immune cells response to modified LPF.

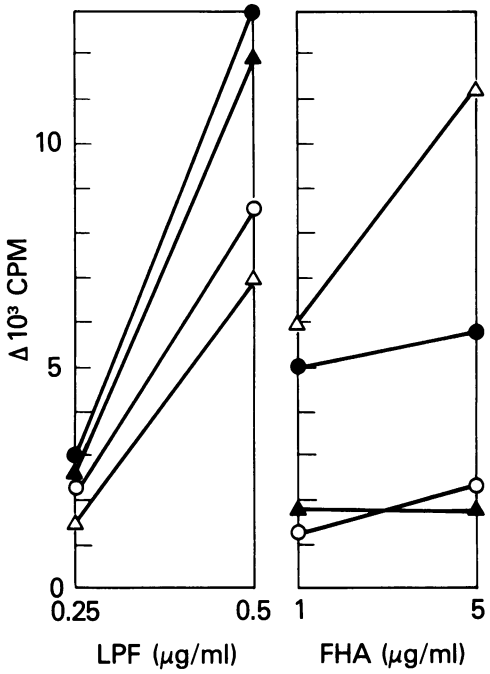


FIG. 3. Specificity of the in vitro proliferative response. Mice were immunized with either whole-cell vaccine, detoxified LPF, or FHA (12 animals in each group). The lymph node cells were harvested after two injections (control mice received saline injections) and were cultured with LPF or FHA. The values presented are the means of six replicate cultures. Symbols: ○, normal cells; ●, pertussis vaccine-immune cells; △, FHA-immune cells; ▲, LPF-immune cells.

## DISCUSSION

Cell-mediated immunity to LPF in vaccinated mice was demonstrated by an in vitro lymphocyte proliferation assay. A variety of in vitro assays for cell-mediated immunity exist today, and most rely on the elaboration of mediators by the responding immune cells, e.g., macrophage migration inhibition factor, lymphotoxin, chemotactic factor, and interferon (3, 4). In vivo correlates of cell-mediated immunity also exist (footpad swelling, skin reactions), but the main disadvantage of most in vitro and in vivo assays is their reliance on secondary cellular systems to produce the measurable biological entity. LPF, as a potent toxin, may interfere with the processing and amplification of the cell-mediated signals. For example, it has been recently shown that LPF inhibits some macrophage functions (B. D. Meade, P. R. Kind, and C. R. Manclark, Res. J. Reticuloendothel. Soc. **43**:64, 1982). The effects that LPF might have on other inflammatory cells are not yet known. The in vitro proliferation assay, as opposed to other correlates of cell-mediated immunity, is widely accepted as a measure of cell-mediated immunity (17) and is the most direct measure of the cellular response since the responding cells later become the proliferating cells (8, 19). The main advantage this method has for studies of immunity to LPF is that LPF does not inhibit T-lymphocyte proliferation in vitro, and therefore its use will not interfere with the interpretation of results. At high concentrations,

TABLE 1. Effect of T-cell removal on the proliferative response to LPF

| Cultured with (μg/ml): | cpm of tritiated thymidine incorporated[a] into: | | | | |
|---|---|---|---|---|---|
| | Untreated lymph node cells | | | T-depleted[b] lymph node cells | |
| | Normal | Immune | SI | Normal | Immune |
| LPF (0.25) | 826 | 73,131 | 88.5 | 91 | 0 |
| LPF (1.0) | 15,310 | 25,678 | 1.7 | 104 | 0 |
| LPF (2.5) | 76,771 | 74,824 | <1.0 | 0 | 0 |
| LPS | 124,936 | 132,996 | | 91,210 | 75,005 |
| ConA | 219,244 | 198,612 | | 170 | 0 |

[a] Average value of six replicated cultures. Cells were collected from six animals per treatment group.
[b] T-cells were depleted by treating the whole lymph node cell population with a monoclonal anti-Thy-1.2 antiserum, followed by agarose-absorbed guinea pig complement (for the complete procedure see reference 7).

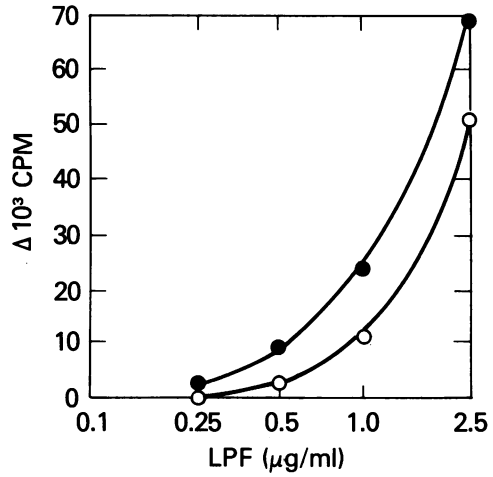


FIG. 4. In vitro proliferative response after immunization with detoxified LPF. A group of 12 mice were immunized with two injections of 5 μg of iodine-detoxified LPF, and their lymph node cells were harvested and cultured. The values presented are the means of six replicate cultures. Symbols: ○, normal cells; ●, immune cells.

LPF is a powerful mitogen of T-cells (10, 14), and from this characteristic stems the problem of the high background of proliferation in LPF-treated cultures. The high background response of normal lymphoid cells is a source of concern whenever specificity and sensitivity are desired.

Specificity is an important characteristic of any immune response. Proof of specificity of an in vitro proliferation will support the notion that this reaction represents an immune response. That the proliferative response of the immune lymph node cells was a demonstration of a specific cell-mediated immunity to LPF was supported by the following: (i) the specificity of the response to the immunizing antigen; (ii) the ability of chemically modified, nonmitogenic LPF to induce proliferation in immune lymph node cells; and (iii) a dependence on T-cells for the demonstration of the proliferative response of immune cells to LPF. We are aware that the elimination of T-cells with Thy-1.2 antibody and complement does not determine whether the T-cells were required accessory cells or were the proliferating cells. However, Kong and Morse (10) provided additional evidence that the mitogenic response to LPF was a proliferation of T-cells, not B-cells. It is highly probable that the proliferative response in immune lymph node cells was also a T-cell proliferative response.

LPF appears to be important in the pathogenesis and immunogenicity of *B. pertussis*. Active immunization with a properly detoxified LPF can protect mice from the standard intracerebral challenge with *B. pertussis* (5, 15, 16, 22). It also has been shown that passively administered antibodies to LPF can protect mice from aerosol or intracerebral challenge with *B. pertussis* (16, 21, 22). Thus, it is interesting that, in this study, mice which received protective doses of whole-cell vaccine did not respond with detectable levels of serum antibody to LPF. The amount of immunologically available LPF in whole-cell vaccine is unknown. Therefore, the differential ELISA antibody response to LPF after vaccination with whole-cell vaccine or inactivated LPF may be the result of quantitative differences in LPF content between whole-cell and purified-antigen vaccines. It should be noted, however, that both immunization protocols confer comparable cell-mediated immunity and protection. There are other antigens of *B. pertussis* that can protect mice from challenge (2, 5, 16), and a serum and/or a cell-mediated immune response to these antigens or both may confer protection. It is also possible that a cell-mediated immune response to LPF plays a role in protection.

TABLE 2. Anti-LPF antibody and cell-mediated immunity after immunization with pertussis vaccine or LPF[a]

| Immunogen[b] | Anti-LPF antibody[c] | Anti-whole-cell antibody[c] | Cell-mediated response to LPF[d] |
|---|---|---|---|
| Saline | 47 ± 16 | 429 ± 186 | |
| Pertussis vaccine lot no. 7b | 51 ± 17 | 1,389 ± 1,000 | 4.5 ± 2.0 |
| LPF | 172 ± 86 | 150 ± 113 | 2.2 ± 0.2 |

[a] All values presented in the table are means ± standard deviation of 50 mice.
[b] See text for immunization schedules and doses.
[c] Optical density × 1,000 U obtained by ELISA and corrected for 1 h of color development.
[d] SI at 0.1 to 0.5 μg/ml.

Although antibodies can protect mice from challenge with *B. pertussis* (16, 21, 22), the mechanisms of cell-mediated immunity in pertussis should be considered an important means for protection against infection and for modifying the disease process. The role of cell-mediated immunity in bacterial diseases has been recently extensively reviewed and evaluated by Hahn and Kaufman (9). Although the full extent of diseases controlled by cell-mediated immunity is not known, it is clear that in some instances it is the most important protective mechanism. Generally, cell-mediated immunity exerts its protection by recruiting and activating other cell types, such as macrophages and granulocytes.

Knowledge of cell-mediated immunity in pertussis may be necessary to establish criteria for the evaluation of the new acellular pertussis vaccines. The absence of a detectable serological response after vaccination may not necessarily mean that vaccination has failed and that the protection has been denied. Proper evaluation of a potential immunogen or of the immune status of vaccinees may require appropriate and technically feasible assays for cell-mediated immunity.

## ACKNOWLEDGMENTS

This work was done while F. Fish was a recipient of a National Institutes of Health and Food and Drug Administration Visiting Fellowship administered by the Fogarty International Center of the National Institutes of Health.

## LITERATURE CITED

1. **Alkan, S. S.** 1979. Assay for antigen specific T-cell proliferation in mice, p. 309–315. *In* I. Lefkovits and B. Pernis (ed.), Immunological methods. Academic Press, Inc., New York.
2. **Ashworth, L. A. E., R. E. Fitzgeorge, L. I. Irons, C. P. Morgan, and A. Robinson.** 1982. Rabbit nasopharyngeal colonization by *Bordetella pertussis*: the effects of immunization on clearance and on serum and nasal antibody levels. J. Hyg. **88**:475–486.
3. **Bloom, B. R., and J. R. David (ed.).** 1976. *In vitro* methods in cell-mediated and tumor immunity. Academic Press, Inc., New York.
4. **Bloom, B. R., and P. R. Glade (ed.).** 1971. *In vitro* methods in cell-mediated immunity. Academic Press, Inc., New York.
5. **Cowell, J. L., Y. Sato, H. Sato, B. An der Lan, and C. R. Manclark.** 1982. Separation, purification, and properties of the filamentous hemagglutinin and the leukocytosis promoting factor-hemagglutinin from *Bordetella pertussis*. Semin. Infect. Dis. **4**:371–379.
6. **Engvall, E., and P. Perlman.** 1972. Enzyme-linked immunosor-

bent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109**:129–135.
7. **Fish, F., and M. Ziff.** 1982. The *in vitro* and *in vivo* induction of anti-double-stranded DNA antibodies in normal and autoimmune mice. J. Immunol. **128**:409–414.
8. **Forbes, J. T., Y. Nakao, and R. T. Smith.** 1975. Tumor specific immunity to chemically induced tumors. Evidence for immunologic specificity and shared antigenicity in lymphocyte responses to soluble tumor antigens. J. Exp. Med. **141**:1181–1200.
9. **Hahn, H., and S. H. E. Kaufman.** 1981. The role of cell mediated immunity in bacterial infections. Rev. Infect. Dis. **3**:1221–1225.
10. **Kong, A. S., and S. I. Morse.** 1977. The *in vitro* effects of *Bordetella pertussis* lymphocytosis-promoting factor on murine lymphocytes. II. Nature of the responding cells. J. Exp. Med. **145**:163–174.
11. **Layne, E.** 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol. **3**:447–454.
12. **Lönnroth, I., and J. Holmgren.** 1975. Protein reagent modification of cholera toxin: characterization of effects on antigenic receptor-binding and toxic properties. J. Gen. Microbiol. **91**:263–277.
13. **Medical Research Council.** 1956. Vaccination against whooping-cough: relation between protection in children and results of laboratory tests. Br. Med. J. **2**:454–462.
14. **Morse, J. H., A. S. Kong, J. Lindenbaum, and S. I. Morse.** 1977. The mitogenic effect of the lymphocytosis-promoting factor from *Bordetella pertussis* on human lymphocytes. J. Clin. Invest. **60**:683–692.
15. **Munoz, J. J., H. Arai, R. K. Bergman, and P. L. Sadowski.** 1981. Biological activities of crystalline pertussigen from *Bordetella pertussis*. Infect. Immun. **33**:820–826.
16. **Munoz, J. J., H. Arai, and R. L. Cole.** 1981. Mouse-protecting and histamine-sensitizing activities of pertussigen and fimbrial hemagglutinin from *Bordetella pertussis*. Infect. Immun. **32**:243–250.
17. **Oppenheim, J. J., and B. Schecter.** 1980. Lymphocyte transformation, p. 233–245. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.
18. **Pittman, M.** 1979. Pertussis toxin: the cause of the harmful effects and prolonged immunity of whooping cough: a hypothesis. Rev. Infect. Dis. **1**:401–412.
19. **Revillard, J. P.** 1982. Delayed hypersensitivity, p. 365–398. *In* J. F. Bach, R. S. Swenson, and R. S. Schwartz (ed.), Immunology, 2nd ed. John Wiley & Sons, Inc., New York.
20. **Sato, Y., J. L. Cowell, H. Sato, D. G. Burstyn, and C. R. Manclark.** 1983. Separation and purification of the hemagglutinins from *Bordetella pertussis*. Infect. Immun. **41**:313–320.
21. **Sato, Y., K. Izumiya, H. Sato, J. L. Cowell, and C. R. Manclark.** 1980. Aerosol infection of mice with *Bordetella pertussis*. Infect. Immun. **29**:261–266.
22. **Sato, Y., H. Sato, K. Izumiya, J. L. Cowell, and C. R. Manclark.** 1982. Role of antibody to filamentous hemagglutinin and to leukocytosis promoting factor-hemagglutinin in immunity to pertussis. Semin. Infect. Dis. **4**:380–385.

22a. **Sekura, R. D., F. Fish, C. R. Manclark, B. D. Meade, and Y. Zhang.** 1983. Pertussis toxin: affinity purification of a new ADP-ribosyltransferase. J. Biol. Chem. **258**:14647–14651.

23. **Standfast, A. F. B.** 1958. The comparison between field trials and mouse protection tests against intranasal and intracerebral challenges with *Bordetella pertussis*. Immunology **1**:135–143.