Reciprocal Protective Immunity against *Bordetella pertussis* and *Bordetella parapertussis* in a Murine Model of Respiratory Infection

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Whooping cough caused by *Bordetella pertussis* is a serious disease in children. Commercial pertussis vaccines, which consist of killed *B. pertussis* cells or derived antigens, are very effective and have reduced the incidence of whooping cough very considerably. However, the protective immunity induced by infection with *B. pertussis* and with *B. parapertussis* was examined in a murine model of respiratory infection. Convalescent mice that had been infected by aerosol with *B. pertussis* or with *B. parapertussis* exhibited a protective immune response against *B. pertussis* and also against *B. parapertussis*. Anti-filamentous hemagglutinin (anti-FHA) serum immunoglobulin G (IgG) and anti-FHA lung IgA antibodies were detected in both mice infected with *B. pertussis* and those infected with *B. parapertussis*. Antibodies against pertussis toxin (anti-PT) and against killed *B. pertussis* cells were detected in mice infected with *B. pertussis*. Pertactin-specific antibodies and antibodies against killed *B. parapertussis* cells were detected in mice infected with *B. parapertussis*. Spleen cells from mice infected with *B. pertussis* secreted interferon-γ (IFN-γ) in response to stimulation by FHA or PT. Spleen cells from mice infected with *B. parapertussis* also secreted IFN-γ in response to FHA. Interleukin-4 was not produced in response to any of the antigens tested. The profiles of cytokine secretion in vitro revealed induction of a Th1-biased immune response during convalescence from infection by *B. pertussis* and by *B. parapertussis*. It is possible that Th1 and Th2 responses against FHA might be related to the reciprocal protection achieved in our murine model.

**MATERIALS AND METHODS**

**Mice.** Specific-pathogen-free female dd-Y mice were obtained from Japan SLC (Hamamatsu, Japan). All mice were 3.5 weeks old at the start of experiments.

**Bacterial strains and culture conditions.** The phase I strain of *B. pertussis* strain 18-323 and *B. parapertussis* strain 23054 were used in this study. Cells were grown on Bordet-Gengou (BG) agar supplemented with 20% (vol/vol) defibrinated horse blood at 37°C.

**Bacterial antigens.** Killed whole-cell *B. pertussis* or *B. parapertussis* antigens were prepared as described below. *B. pertussis* or *B. parapertussis* was cultured on BG plates for 30 h at 37°C. Cells were harvested in phosphate-buffered saline (PBS) on ice, and suspensions of cells were adjusted to 10^10 cells/ml after measurement of the optical density at 660 nm (OD660) of the suspension. The bacterial suspension was supplemented with formalin to a final concentration of 0.2 M. After incubation for 1 h at 37°C, the suspension of formalin-killed whole cells was supplemented with 0.2 M lysine and then it was dialyzed against PBS for 2 days at 4°C.

**FHA and pertussis toxin (PT)** were purified from the culture supernatant of *B. pertussis* by modified versions of the methods of Menozzi et al., Chong and Klein, and Sekura et al. (5, 17, 26, 30). PRN was purified from a heated extract of *B. pertussis* cells by a modified version of the method of Gould-Kostka et al. (7). Purified FHA, PT, and PRN were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a modified version of Laemmli’s method (14). No contaminants were detected in each purified preparation (data not shown). Detoxified pertussis toxin (PTd) was prepared as described previously (31).
Aerosol infection. Infection by aerosols of B. pertussis or B. parapertussis was achieved by modified version of the method of Oda et al. (21, 30, 31). B. pertussis or B. parapertussis was cultured on BG plates for 30 h at 37°C. The bacteria were then harvested in PBS on ice and then each suspension of bacteria was adjusted to 10^9 CFU/ml after measurement of the OD_600. Mice were allowed to inhale the suspension for 45 min in a sealed aerosol chamber within a biosafety cabinet (MHE-130B; Sanyo Electric, Moriguchi, Japan). The number of viable Bordetella cells in each mouse lung after such treatment was approximately 10^7 CFU.

Quantitation of bacteria in lungs. After sacrifice, the lungs of mice were dissected and homogenized in 10 ml of PBS per lung in a Teflon homogenizer on ice. After dilution of each lung homogenate, it was spread on BG plates and incubated for 4 days at 37°C. The number of CFU was used to estimate the number of viable bacteria. The limit of detection was 10^3 CFU/lung by this method (31).

Assay of protective immunity. Protective immunity was demonstrated as described previously (30, 31). Convalescent mice, which were maintained in individual cages for 6 weeks after primary infection with an aerosol of B. pertussis or B. parapertussis, were infected via the respiratory tract by an aerosol of B. pertussis or B. parapertussis. Two weeks after the second infection, the lungs of each mouse were surgically removed and homogenized in PBS in a Teflon homogenizer on ice. The number of CFU was measured as described above. The significance of the difference between the nonimmunized control group and each immunized (convalescent) group was examined by Student’s t test. Probability values of <0.05 were considered evidence of statistical significance (30, 31).

Quantitation of antibodies by enzyme-linked immunosorbent assays (ELISAs). Sera and lungs of convalescent mice which had been maintained for 6 weeks after the first infection with B. pertussis or B. parapertussis were obtained for determinations of levels of antigen-specific serum immunoglobulin G (IGG) and lung IgA antibodies. Lungs were homogenized in 10 ml of PBS per lung that contained 0.1 mM phenylmethylsulfonyl fluoride and 8% (vol/vol) fetal calf serum in a Teflon homogenizer on ice. Homogenates were centrifuged (25,000 rpm, 30 min, 4°C), and supernatants were used for determinations of levels of antigen-specific IgA antibodies in lungs (24).

FHA- and PT-specific antibodies were quantitated by ELISAs, as described previously (30, 31). Levels of antibodies were expressed in terms of mean absorbance at 492 nm (OD_492).

Levels of PRN-specific antibodies were determined by a modified version of the ELISA method of Manghi et al. (16) and were expressed as mean absorbance values at 405 nm (OD_405).

Assay of FHA-neutralizing antibodies. Samples of serum from mice infected with B. pertussis and mice infected with B. parapertussis were obtained and diluted serially with PBS. Aliquots of 50 µl of each dilution were mixed with a solution of purified FHA that contained 16 hemagglutinating units of purified FHA. After incubation for 1 h at 37°C, each sample was supplemented with 50 µl of a suspension of chicken erythrocytes (0.5% [vol/vol]). After mixing and incubation for 2 h at room temperature, the maximum dilution at which hemagglutination was inhibited was taken as the titer of FHA-neutralizing antibodies.

Agglutination test. Levels of serum antibodies against B. pertussis cells or B. parapertussis cells were determined by an agglutination test (25).

Samples of serum from convalescent mice were serially diluted in 96-well round-bottom plates (50 µl/well). Then 50 µl of the suspension of killed whole cells (10^10 cells/ml) was added to each well. After mixing and incubation for 2 days at 4°C, the maximum dilution that induced agglutination was taken as the titer of specific antibodies.

Proliferation of spleen cells. Proliferation of spleen cells upon stimulation by B. pertussis or B. parapertussis antigens was examined by a modified version of the method of Ahmed et al. (1). Splenocytes were surgically and aseptically removed from convalescent mice. Single-cell suspensions (10^6 cells/ml) of splenocytes were prepared in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, 50 µg of gentamicin (Life Technologies, Rockville, Md.), and 10 µg of polymyxin B (Sigma, St. Louis, Mo.). In order to neutralize the cytokine-inducing activity of the endotoxin in the preparation of whole-cell antigens, the medium was supplemented with polymyxin B (3, 6). The suspensions of spleen cells were supplemented with FHA (5 µg/ml), PTd (5 µg/ml), PRN (5 µg/ml), and killed whole B. pertussis cells (10^6 cells/ml) or killed whole B. parapertussis cells (10^6 cells/ml). Then, 200-µl aliquots of the mixture that contained spleen cells or antigens were placed in wells of a flat-bottom 96-well plate. Control wells contained the spleen cells but no antigens for stimulation. Blank wells contained medium only (no spleen cells and no antigens for stimulation). After incubation of the plate in a CO_2 incubator (5% CO_2 in air) for 24 h at 37°C, 20 µl of Alamar Blue (AccuMed International, Chicago, Ill.) was added to each well and the plate was incubated for 48 h under the same conditions. The OD_700 and OD_570 of the contents of each well were measured with a microplate reader (MTP-120; Corona Electric, Hachiinaka, Japan). A proliferation index (PI) was calculated from the following formula: PI = ([OD_700 - OD_570]blank - [OD_700 - OD_570]sample) / [OD_700 - OD_570]blank. The plate was incubated for 48 h under the same conditions. The OD_700 and OD_570 of the contents of each well were measured with a microplate reader (MTP-120; Corona Electric, Hachiinaka, Japan). A proliferation index (PI) was calculated from the following formula: PI = ([OD_700 - OD_570]blank - [OD_700 - OD_570]sample) / [OD_700 - OD_570]blank.

Secretion of cytokines by spleen cells in vitro. Spleen cells of convalescent mice were tested in vitro for secretion of cytokines in response to B. pertussis or B. parapertussis antigens by a modified version of the method of Redhead et al. (23).

Single-cell suspensions of spleen cells (2.0 × 10^6 cells/ml) were incubated with FHA (5 µg/ml), PTd (5 µg/ml), PRN (5 µg/ml), and killed whole B. pertussis cells (10^6 cells/ml) or killed whole B. parapertussis cells (10^6 cells/ml) at 37°C for 72 h in an atmosphere of 5% CO_2 in air. Culture supernatants were obtained by centrifugation (450 × g, 5 min, 4°C) and stored at −80°C prior to assays. Mouse gamma interferon (IFN-γ) and mouse interleukin-4 (IL-4) were quantitated by ELISAs. A commercial Cytoscreen Immunoassay Kit (BioSource International Inc., Camarillo, Calif.) was used according to the manufacturer’s recommendations.

Quantitation of protein. Proteins were quantitated by Bradford’s method (4) with egg albumin (Sigma) as the standard protein.

Statistical analysis. The statistical significance of differences between results from different groups was examined by Student’s t test.

RESULTS

Time course of numbers of bacterial cells in mouse lungs after primary infection with B. pertussis or with B. parapertussis. Mice were infected with B. pertussis or with B. parapertussis as described in Materials and Methods. Initial counts of viable bacteria in lungs of mice infected with B. pertussis and with B. parapertussis were 10^4.8 and 10^5.3 CFU/lung, respectively (Fig. 1). There was no significant difference between these values (P > 0.05). Numbers of bacteria increased for 1 week after infection and then declined slowly. The mice recovered within 6 weeks of infection, at which time no bacteria were detected in their lungs (Fig. 1). These mice were taken as the convalescent groups for this study. There were no significant differences in terms of CFU in lungs at each time point between mice infected with B. pertussis and those infected with B. parapertussis.

Protective effects of previous infection against B. pertussis. Mice infected with B. pertussis or mice infected with B. para-
B. pertussis antibody responses against control group and those for each convalescent group (\(P < 0.05\)). There were significant differences between the results for the control group and each convalescent group (\(P < 0.05\)). The results demonstrated that protection against B. pertussis in infection not only with B. pertussis but also with B. parapertussis (BPP) was established during convalescence from infection by B. pertussis or by B. parapertussis.

Protective effects of previous infection against B. parapertussis. Since we had found that mice that had recovered from infection not only with B. pertussis but also with B. parapertussis exhibited protective immunity against B. pertussis, we next examined protection against B. parapertussis. We found that protective immunity against B. parapertussis was induced in mice infected with B. pertussis and in those infected with B. parapertussis (Fig. 2B). The numbers of CFU in lungs of control mice, mice infected with B. pertussis, and mice infected with B. parapertussis were 10^{2.7}, 10^{2.1}, and 10^{2.0}\text{CFU/lung}, respectively. There were significant differences between the results for the control group and those for each convalescent group (\(P < 0.05\)).

Levels of antibodies in convalescent mice. We examined the antibody responses against B. pertussis and B. parapertussis antigens and found FHA-specific serum IgG antibodies not only in mice infected with B. pertussis but also in mice infected with B. parapertussis 6 weeks after primary infection (Fig. 3A). These antibodies were not detected in control mice. There was no significant difference between the levels of FHA-specific antibodies in mice infected with B. pertussis and in mice infected with B. parapertussis (\(P > 0.05\)). The calculated titer of FHA-neutralizing antibodies in the serum of both convalescent groups was 1,024. As shown in Fig. 3B, a significant level of PT-specific serum IgG was produced only by mice infected with B. pertussis (\(P < 0.05\)), and PRN-specific IgG was detected in the sera of mice infected with B. parapertussis but not in those of mice infected with B. pertussis (Fig. 3C). The titer in the agglutination test against killed B. pertussis cells was high for the sera of mice infected with B. pertussis but not for sera of mice infected with B. parapertussis (Fig. 4A). A high level of PRN-specific antibodies in mice infected with B. parapertussis but not with B. pertussis was noted.

FIG. 2. Protection of convalescent mice against B. pertussis or B. parapertussis. Control mice, mice infected with B. pertussis (BP), and mice infected with B. parapertussis (BPP) were challenged by exposure to an aerosol of B. pertussis (A) or B. parapertussis (B). Two weeks after the challenge, numbers of B. pertussis cells or B. parapertussis cells in each mouse lung were counted. The results shown are mean values per lung, as estimated from individual lungs of five mice for each group. Each column and vertical line represent a mean and standard error. *, \(P < 0.05\) versus the control group.

FIG. 3. Antigen-specific serum IgG responses in mice infected with B. pertussis and mice infected with B. parapertussis. Sera of control mice, mice infected with B. pertussis (BP), and mice infected with B. parapertussis (BPP) were examined by ELISAs. Levels of antibodies were expressed as mean values of absorbance at the indicated wavelength. (A) FHA-specific serum IgG. (B) PT-specific serum IgG. (C) PRN-specific serum IgG. Each column with a vertical line represents a mean and standard error of results from individual samples of serum from five mice for each group. *, \(P < 0.05\) versus the control group.

FIG. 4. Whole-cell agglutination titers in mice infected with B. pertussis and mice infected with B. parapertussis. Sera of control mice, mice infected with B. pertussis (BP), and mice infected with B. parapertussis (BPP) were examined as described in Materials and Methods. The titers of antibodies are expressed as mean values. (A) Antibodies against B. pertussis. (B) Antibodies against B. parapertussis. See legend to Fig. 3 for details.
agglutinating antibodies against *B. parapertussis* was found only in the sera of mice infected with *B. parapertussis* (Fig. 4B).

We detected a response in terms of FHA-specific lung IgA both in mice infected with *B. pertussis* and in mice infected with *B. parapertussis* (Fig. 5A). Mice infected with *B. parapertussis* produced lower levels of the antibodies than mice infected with *B. pertussis* but the difference from control mice was significant (*P* < 0.05). Furthermore, PT-specific lung IgA was produced only by mice infected with *B. pertussis* (Fig. 5B). No PRN-specific lung IgA was detected in any samples examined (Fig. 5C).

**Responses of spleen cells to *B. pertussis* and to *B. parapertussis* antigens.** We examined the proliferative responses in vitro of antigen-stimulated spleen cells from mice infected with *B. pertussis* and mice infected with *B. parapertussis*. The proliferation of spleen cells of mice infected with *B. pertussis* was induced to a greater or lesser extent upon stimulation with FHA, PTd, and killed *B. pertussis* cells (Fig. 6). In the case of spleen cells from mice infected with *B. parapertussis*, proliferation was induced by FHA and by killed *B. parapertussis* cells, but not by PTd. No PRN-specific proliferative response was detected in either convalescent group.

As shown in Fig. 7, spleen cells derived from mice infected with *B. pertussis* secreted significant levels of IFN-γ in response to stimulation in vitro by FHA or by PTd (*P* < 0.05). Stimulation by PRN, killed whole *B. pertussis* cells, or killed whole *B. parapertussis* cells did not induce any significant secretion of IFN-γ from the spleen cells of mice infected with *B. pertussis* (*P* > 0.05). Spleen cells from mice infected with *B. parapertussis* secreted IFN-γ upon stimulation by FHA (*P* < 0.05). Although some secretion of IFN-γ was detected upon stimulation by killed *B. parapertussis* cells, the level was not significant (*P* > 0.05). There was no significant difference in terms of secretion of IFN-γ between the spleen cells of mice infected with *B. parapertussis* and those of control mice after stimulation of spleen cells with PTd, PRN, or killed *B. pertussis* cells.

The spleen cells of mice infected with *B. pertussis* and of mice infected with *B. parapertussis* did not release IL-4 upon stimulation by any of the antigens that we tested (data not shown).

**DISCUSSION**

Our data provide a demonstration of the relationship between the protective immunity induced by natural infection with *B. pertussis* and that induced by infection with *B. parapertussis*. We demonstrated that reciprocal protection is induced in mice infected with *B. pertussis* and those infected with *B. parapertussis* in a murine model of respiratory infection. Stehr et al. reported that the commercial pertussis vaccine prepared from antigens of *B. pertussis* had no or little efficacy against *B. parapertussis* in children (27). Khelif et al. suggested that reciprocal immunity might not be established between the two species of *Bordetella* in a murine model of respiratory infection (13). In their experiment, children or mice were immunized by subcutaneous or peritoneal injection. Immunization by injection of antigens effectively induces a serum antibody response. However, it does not effectively activate mucosal and Th1
responses, which are important for protection against *B. pertussis* (12, 19, 20, 23). Furthermore, Mills et al. suggested a difference in terms of protective immune responses between mice that have recovered from infection by *B. pertussis* and mice that have been vaccinated by injection (20). Thus, the method of immunization might be important for detection of the reciprocal immune responses induced by infection with *B. pertussis* and *B. parapertussis*.

We detected increases in levels of FHA-specific serum IgG and FHA-specific lung IgA in mice infected with *B. pertussis* and mice infected with *B. parapertussis*. Furthermore, we detected FHA-neutralizing antibodies in the sera not only of mice infected with *B. pertussis* but also of mice infected with *B. parapertussis*. It is known that both these species of *Bordetella* produce FHA, and the antigenicities of the FHAs are similar (13). Moreover, FHA is important for the attachment to host cells in the case of both *B. pertussis* and *B. parapertussis* (28).

He et al. suggested that antibodies against FHA of *B. parapertussis* in serum IgG might be responsible for protection against *B. parapertussis* in outbreaks of infection in West Finland (8). Thus, previous reports and our data suggest that FHA-specific antibodies in sera and lungs might be important in the establishment of reciprocal protection. We detected PT-specific serum IgG and lung IgA antibodies in mice infected with *B. pertussis* but not in mice infected with *B. parapertussis*. The genome of *B. parapertussis* includes a *ptx* gene but this gene is not transcribed because of mutations in the promoter region (2). PT is a major protective antigen against *B. pertussis* infection and an important component of pertussis vaccines. Although PT should be included in pertussis vaccines, PT cannot function for protection against *B. parapertussis*. We found no PRN-specific serum IgG and no PRN-specific lung IgA in the mice infected with *B. pertussis* in this study, confirming the reports of Mills et al. (19) and Redhead et al. (23). It is thought that PRN-specific serum antibodies played a minor role in the protection against *B. pertussis* in our convalescent mice. However, we detected an increase in the level of PRN-specific serum IgG antibodies in our mice infected with *B. parapertussis*, which might indicate the importance of PRN in the protective immune response in mice infected with *B. parapertussis*. No PRN-specific IgA antibodies were produced in the lungs of mice infected with *B. pertussis* or of mice infected with *B. parapertussis*. In both groups of convalescent mice, the mucosal immune response to PRN might have played only a minor protective role.

In our presentation of the results of agglutination tests, we did not detect cross-reactions between the sera from mice infected with *B. pertussis* and those from mice infected with *B. parapertussis*. There are considerable immunological differences in terms of surface structures, which include fimbriae, between *B. pertussis* and *B. parapertussis*. Willems et al. reported that type-2 and type-3 fimbriae from *B. pertussis* partially protected mice against infection by *B. parapertussis* (32). They postulated that the partial protection against *B. parapertussis* infection might have been based on structural differences between the cell surfaces, including fimbriae. It has been suggested that the antibodies against fimbriae might be responsible for reciprocal protection (32). Nevertheless, cross-reaction in the agglutination test was not detected in our convalescent mice. Although agglutinating antibodies against *B. pertussis* might have a role in protection against *B. pertussis* infection, the role of the agglutinating antibodies in reciprocal protection might have been minor.

It has been reported that Th1 responses play a major role in protection against *B. pertussis* in convalescent mice (19, 20, 23). We confirmed the induction of Th1 responses in mice infected with *B. pertussis*. In the case of mice infected with *B. pertussis*, proliferation of spleen cells in vitro was detected after stimulation with FHA and PT. The stimulation of the cells by FHA and PT induced the secretion of IFN-γ, but not of IL-4. In the case of spleen cells from mice infected with *B. parapertussis*, both the proliferation of spleen cells and the secretion of IFN-γ were detected after stimulation with FHA. However, the amount of IFN-γ secreted after stimulation by FHA from spleen cells of mice infected with *B. parapertussis* was lower than that from spleen cells of mice infected with *B. pertussis*. The results might be explained by differences in epitopes, recognized by the system for Th1, between FHA of *B. pertussis* and that of *B. parapertussis*. No secretion of IL-4 from spleen cells of mice infected with *B. pertussis* or of mice infected with *B. parapertussis* was detected. Our results reveal the induction of a Th1-biased immune response in mice infected with *B. parapertussis*, as well as in mice infected with *B. pertussis*. In this study, both mice infected with *B. pertussis* and mice infected with *B. parapertussis* exhibited a Th1 response against FHA, a result that might suggest the participation of Th1 responses against FHA in reciprocal protection.

No proliferation and no secretion of IFN-γ and IL-4 were detected upon stimulation by PRN of spleen cells from mice infected with *B. pertussis* and from mice infected with *B. parapertussis*. PRN might have played only a minor role on Th1 responses in our convalescent mice.

We observed proliferative responses against killed *B. pertussis* cells and killed *B. parapertussis* cells by spleen cells of mice infected with *B. pertussis* and of mice infected with *B. parapertussis*, respectively. The results suggest that cell surface antigens of *B. pertussis* and *B. parapertussis* might play a role in Th1 responses against *B. pertussis* and *B. parapertussis*, respectively. However, it is unlikely that Th1 responses against cell surface antigens play a major role in reciprocal protection because of differences in the antigenicity of the cell surface between *B. pertussis* and *B. parapertussis*.

For this study, we used antigens produced exclusively by *B. pertussis*, with the exception only of killed *B. parapertussis* cells. Further experiments are necessary to clarify virulence and immunological relationships between *B. pertussis* and *B. parapertussis* using the corresponding antigens produced by *B. parapertussis*. The virulence factors of *B. parapertussis*, including FHA, PRN, and fimbriae, have not been fully characterized in terms of biological activity and antigenicity. We are now characterizing the virulence factors of *B. parapertussis* and comparing them with those of *B. pertussis*. It is also necessary to confirm that our data in a murine model of respiratory infection correlate with the data in humans.

*B. parapertussis*, as well as *B. pertussis*, causes whooping cough. Although pertussis vaccines decrease the incidence of whooping cough, several studies reported that the pertussis vaccines have no or limited efficacy against *B. parapertussis* (9, 13, 15, 27, 32). In order to prevent whooping cough caused by the two species, pertussis vaccines should have sufficient effi-
cacy against not only B. pertussis but also B. parapertussis. In this study, we have shown that reciprocal immunity can be induced between B. pertussis and B. parapertussis in a murine model of respiratory infection. It appears that Th1 and Th2 responses against FHA may be related to this reciprocal protection. It is thought that the data are important for the study of a vaccine which is effective against not only B. pertussis but also B. parapertussis. Studies of immunization which induce reciprocal protection against B. pertussis and B. parapertussis are proceeding in our laboratories.

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REFERENCES