Role of Systemic and Mucosal Immune Responses in Reciprocal Protection against *Bordetella pertussis* and *Bordetella parapertussis* in a Murine Model of Respiratory Infection

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The roles of systemic humoral immunity, cell-mediated immunity, and mucosal immunity in reciprocal protective immunity against *Bordetella pertussis* and *Bordetella parapertussis* were examined by using a murine model of respiratory infection. Passive immunization with serum from mice infected with *B. pertussis* established protective immunity against *B. pertussis* but not against *B. parapertussis*. Protection against *B. parapertussis* was induced in mice that had been infected with serum from mice infected with *B. parapertussis* but not from mice infected with *B. pertussis*. Adoptive transfer of spleen cells from mice infected with *B. pertussis* or *B. parapertussis* also failed to confer reciprocal protection. To examine the role of mucosal immunity in reciprocal protection, mice were infected with preparations of either *B. pertussis* or *B. parapertussis*, each of which had been incubated with the bronchoalveolar wash of mice that were convalescing after infection with *B. pertussis* or *B. parapertussis*. Such incubation conferred reciprocal protection against *B. pertussis* and *B. parapertussis* on infected mice. The data suggest that mucosal immunity including secreted immunoglobulin A in the lungs might play an important role in reciprocal protective immunity in this murine model of respiratory infection.

MATERIALS AND METHODS

**Mice.** Specific-pathogen-free female dd-Y and BALB/c (H-2\(^d\)) mice were obtained from Japan SLC (Hamamatsu, Japan). The mice were maintained under specific-pathogen-free conditions. All mice were 4 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 at 37°C on Bordet-Gengou (BG) agar supplemented with 20% (vol/vol) defibrinated horse blood.

**Aerosol infection.** Infection by an aerosol of *B. pertussis* or *B. parapertussis* was achieved by a previously described method (19–21). *B. pertussis* and *B. parapertussis* were cultured separately on BG plates for 30 h at 37°C. The cells were then harvested in phosphate-buffered saline (PBS) on ice, and each suspension of cells was adjusted to 10\(^3\) cells/ml after measurement of the optical density at 660 nm. Four-week-old mice were allowed to inhale the suspension for 45 min in a sealed aerosol chamber within a biosafety cabinet (MHE-130B1; Sanyo Electric, Moriguchi, Japan). The number of viable *Bordetella* cells in each mouse lung after such treatment was approximately 10\(^5\) CFU. Convalescent mice were maintained in individual cages for 6 weeks after infection with *B. pertussis* or *B. parapertussis*.

**Quantitation of bacteria in lungs.** After sacrifice, the lungs of mice were dissected out and homogenized in 10 ml of PBS per lung in a Teflon homogenizer on ice. Serial dilutions of each lung homogenate were spread on BG plates, and cells were cultured for 4 days at 37°C. The number of CFU was used to estimate the number of bacteria. The limit of detection of this method was 10\(^2\) CFU/lung (19–21).

**Assay of protective immunity.** Protective immunity was determined as described previously (20). Mice were infected via the respiratory tract by an aerosol of *B. pertussis* or *B. parapertussis*. Two weeks after infection, the lungs of each mouse were surgically removed and homogenized in PBS in a Teflon homogenizer on ice. The number of CFU was determined as described above.

**Passive transfer of serum.** Serum of nonimmunized control dd-Y mice and of convalescent mice, which had been maintained for 6 weeks after infection with *B. pertussis* or *B. parapertussis*, was prepared for passive immunization. Aliquots of serum (0.4 ml) were injected intravenously into individual 10-week-old dd-Y mice 4 h before aerosol challenge for the assay of protective immunity (12).

**Adaptive transfer of spleen cells.** Adoptive transfer of spleen cells was performed by a modified version of the method of Mills et al. (12). Convalescent BALB/c mice that had been maintained for 6 weeks after infection with *B. pertussis* or *B. parapertussis* were used as donors of “immune” spleen cells. Spleens were removed surgically and aseptically from controls and from conv-
lescent mice, and then single-cell suspensions were prepared. Single-cell suspensions, including cells equal to one spleen from a control mouse or a convalescent mouse, were injected intraperitoneally into recipient 10-week-old BALB/c mice that had been irradiated with sublethal dose of 6 Gy of total body irradiation. After the transfer of spleen cells, the immunity of each mouse to B. pertussis and B. parapertussis was determined as described above.

Bronchoalveolar washes. Bronchoalveolar washes of convalescent and control ddY mice were collected by washing excised trachea and lungs three times with 300 μl of PBS. Approximately 700 μl of bronchoalveolar wash was recovered from each mouse (1). There was little or no contamination with blood, as judged by the hemoglobin content of each wash. The bronchoalveolar washes from mice in individual groups were pooled and stored at −25°C prior to use.

Determination of the protective effect of the bronchoalveolar wash. After B. pertussis or B. parapertussis cells had been cultured on BG plates for 30 h at 37°C, they were harvested in PBS on ice. Individual suspensions of cells were adjusted to 10⁶ cells/ml. Five milliliters of suspension was added to 5 ml of pooled bronchoalveolar wash from convalescent mice that had previously been infected with B. pertussis or with B. parapertussis. Then the mixture was incubated for 30 min at 37°C. The pooled bronchoalveolar washes from noninfected mice were used as the control bronchoalveolar washes. The number of CFU in each suspension was not reduced by this incubation. Four-week-old ddY mice were then allowed to inhale the suspension that had been incubated with the bronchoalveolar wash. The number of viable Bordetella cells in each mouse lung after such treatment was approximately 10⁷ CFU. Two weeks after infection, the CFU in lungs were counted as described above.

Quantitation of antibodies against whole cells of B. pertussis and of B. parapertussis. Preparations of killed-whole-cell B. pertussis or B. parapertussis antigens were prepared with formalin as described previously (20). Cells were suspended in 0.05 M carbonate buffer, pH 9.4 (3 × 10⁵ cells/ml). The 100-μl aliquots of a suspension of killed whole cells of B. pertussis or B. parapertussis were placed in individual wells of 96-well flat-bottom AquaBind plates (Asahi Techno Glass Co., Tokyo, Japan) (4). After incubation for 30 min at 37°C, the plates were washed three times with 300 μl of 0.5 M NaCl that contained 1% (vol/vol) Triton X-100 and each well was coated with 300 μl of 0.05 M carbonate buffer that contained 15% (wt/vol) polyethylene glycol 4000 (Sigma, St. Louis, Mo.), 1% (wt/vol) bovine serum albumin (Innontogen Corp., Carlsbad, Calif.), and 10 mM ethanalamine. After overnight incubation at room temperature, the plate was washed three times with PBS that contained 0.1% (vol/vol) Tween 20 (PBS-TW). Then 100 μl of 50-fold-diluted serum or lung homogenate from a convalescent mouse was added to each well. The plate was then incubated for 1 h at 37°C and washed four times with PBS-TW. Next, 100 μl of 1,000-fold-diluted peroxidase-conjugated antibodies raised in goats against mouse immunoglobulin G (IgG) or IgA (Sigma) was added to each well. The plate was incubated for 1 h at 37°C and washed four times with PBS-TW. Then 100 μl of 3,3′,5,5′-tetramethylbenzidine solution (Pierce, Rockford, Ill.) was placed in each well, and the plate was incubated for 20 min at room temperature. The reaction was stopped by the addition of 100 μl of 1 M sulfuric acid. The absorbance of the solution in each well was measured at 450 nm. The antibody response was expressed as the mean absorbance from four samples (optical density at 450 nm).

Statistical analysis. The statistical significance of differences between results from different groups was examined by Student’s t test. Probability values of <0.05 were considered evidence of statistical significance.

RESULTS

Protective effects of the passive transfer of serum. Mice injected with serum from mice infected with B. pertussis and mice injected with serum from mice infected with B. parapertussis were challenged with an aerosol of B.pertussis. Two weeks after the challenge, the number of CFU in the lungs of each mouse was determined as described in Materials and Methods. The number of CFU in the lungs of control mice was approximately 10⁶.6 (Fig. 1A). For mice injected with serum from mice that had been infected with B. pertussis and for mice injected with serum from mice that had been infected with B. parapertussis, the numbers of CFU in lungs were approximately 10⁵,5 and 10⁶,6, respectively (Fig. 1A). There was a significant difference between the results for the control group and those for mice that had been passively immunized with B. pertussis-specific antiserum. No difference between the results for the control group and those for mice that had been passively immunized with B. parapertussis-specific antiserum was detected. The data suggested that protection against B. pertussis had been established by passive immunization with serum from mice infected with B. pertussis but not with serum from mice infected with B. parapertussis.

We next examined protection against B. parapertussis. Protective immunity against B. parapertussis was detected in mice that had been immunized with serum from mice infected with B. parapertussis but not in mice that had been immunized with serum from mice infected with B. pertussis (Fig. 1B). The numbers of CFU in lungs of control mice, mice immunized with B. pertussis-specific antiserum, and mice immunized with B. parapertussis-specific antiserum were approximately 10⁵,9, 10⁵,5, and 10⁶,5, respectively. There was a significant difference between the results for the control group and the results for mice that had been passively immunized with B. parapertussis-specific antiserum. No difference between the results for the control group and those for mice that had been passively immunized with B. pertussis-specific antiserum was detected. The data indicate that protection against B. parapertussis was established by passive immunization with serum from mice infected with B. parapertussis but not by passive immunization with serum from mice infected with B. pertussis.
Protective effects of the adoptive transfer of spleen cells. For the experiment involving adoptive transfer of spleen cells, we used inbred mice (BALB/c mice), which are suitable for this procedure. Spleen cells from nonimmune control mice or from convalescent mice, which had been infected with B. pertussis or with B. parapertussis, were injected into recipient mice. Two weeks after recipient mice had been challenged with B. pertussis (A) or B. parapertussis (B). Two weeks later, the numbers of CFU in the lungs of recipient mice were counted. The results shown are mean values per lung, as estimated from individual lungs of four mice in each group, plus standard deviations. *, P < 0.05 versus the control group.

FIG. 2. Adoptive transfer of immune spleen cells for protection of recipient mice against B. pertussis and B. parapertussis. Mice were injected with spleen cells from nonimmune control mice (control), from mice infected with B. pertussis (BP), and from mice infected with B. parapertussis (BPP) and then challenged with an aerosol of B. pertussis (A) or B. parapertussis (B). Two weeks later, the numbers of CFU in the lungs of recipient mice were counted. The results shown are mean values per lung, as estimated from individual lungs of four mice in each group, plus standard deviations. *, P < 0.05 versus the control group.

were 10^5.8, 10^5.4, and 10^5.1, respectively (Fig. 2B). There was a significant difference between the control group and the group injected with spleen cells from mice that had been infected with B. parapertussis (P < 0.05).

These data suggest that cell-mediated immunity, which was transferred to recipients by injection of spleen cells from mice that had been infected with B. pertussis, had a protective effect against B. pertussis but not against B. parapertussis.

Role of mucosal immunity in protection. To determine the role of mucosal immunity in reciprocal protection, we infected mice with an aerosol of B. pertussis or B. parapertussis cells that had been incubated with the pooled bronchoalveolar washes of nonimmune control mice or of immune mice, as described in Materials and Methods. Viable B. pertussis cells were incubated for 30 min at 37°C with the pooled bronchoalveolar washes of nonimmune control mice, of mice infected with B. pertussis, or of mice infected with B. parapertussis. The number of CFU in each suspension of cells was not reduced by such incubation, and there were no significant differences among the numbers of CFU in these suspensions. Four-week-old dd-Y mice were allowed to inhale an aerosol of each suspension. Initially, the numbers of viable bacteria in the lungs of all the mice were very similar (approximately 10^6.7 CFU). Two weeks after the aerosol challenge, we counted the CFU in the lungs. In the group that had inhaled B. pertussis that had been incubated with the bronchoalveolar wash of nonimmune control mice, the number of CFU was approximately 10^6.8 (Fig. 3A). There was no significant difference between the results for the control group and the group of mice infected with B. parapertussis. The number of CFU in the mice that had inhaled cells incubated with the bronchoalveolar wash of mice infected with B. pertussis and of mice infected with B. parapertussis were approximately 10^6.0 and 10^5.8, respectively (Fig. 3A). There was a significant difference between the results for the control group and the results for each experimental group (P < 0.05). The results suggest that protection against B. pertussis was established by mucosal antibodies induced during convalescence from infection by B. pertussis or by B. parapertussis.

We next examined the role of mucosal immunity in protection against B. parapertussis. Viable B. parapertussis cells were incubated with the pooled bronchoalveolar washes of nonimmune control mice, of mice infected with B. pertussis, or of mice infected with B. parapertussis and then challenged with the preparation of B. parapertussis. During incubation of viable B. parapertussis cells with the various bronchoalveolar washes, the number of CFU in each suspension remained unchanged, and there were no significant differences among the initial numbers of CFU in the lungs of mice in each group (approximately 10^5.2 CFU). The numbers of CFU of B. parapertussis in mice that had inhaled cells exposed to the bronchoalveolar wash of nonimmune control mice, of mice infected with B. pertussis, and of mice infected with B. parapertussis were approximately 10^6.0, 10^5.1, and 10^5.6, respectively, 2 weeks after the challenge (Fig. 3B). There was a significant difference between the results for the control group and each experimental group (P < 0.05).

Levels of antibodies against B. pertussis and against B. parapertussis. We examined the antibody responses against whole cells of B. pertussis and of B. parapertussis by enzyme-linked immunosorbent assays. As shown in Fig. 4A, significant levels of IgG antibodies against whole cells of B. pertussis were detected only in the serum of mice infected with B. pertussis (P <
and in mice infected with \textit{B. parapertussis} in a murine model of respiratory infection (20). Our observations suggested the possible development of a vaccine that would be effective not only against \textit{B. pertussis} but also against \textit{B. parapertussis}. In the present study, we examined the roles of the systemic humoral immune response, the cell-mediated immune response, and the mucosal immune response in reciprocal protection in our murine model of respiratory infection.

To characterize the role of serum antibodies in reciprocal protective immunity, we examined the protection against \textit{B. pertussis} in mice injected with serum of mice that had been infected with \textit{B. pertussis} or with \textit{B. parapertussis}. We found that serum from mice infected with \textit{B. pertussis} conferred significant protection against \textit{B. pertussis} but not against \textit{B. parapertussis}. Serum from mice infected with \textit{B. parapertussis} protected mice against \textit{B. parapertussis} but not against \textit{B. pertussis}. These data suggest that serum antibodies from convalescent mice do not play a significant role in reciprocal protection against \textit{B. pertussis} and \textit{B. parapertussis}. It is known that pertussis vaccines that are administered by injection induce a serum antibody response for the most part (8). Our results suggest that pertussis vaccines are not useful for protection against \textit{B. parapertussis} infection.

The adoptive transfer of spleen cells from convalescent mice revealed that mice injected with spleen cells from mice in-

**DISCUSSION**

Both \textit{B. parapertussis} and \textit{B. pertussis} cause whooping cough. Pertussis vaccines have decreased the incidence of whooping cough, but they may have little or no efficacy against \textit{B. parapertussis} (6, 7, 9, 10, 17, 22). For the prevention of whooping cough caused by both species of \textit{Bordetella}, pertussis vaccines should protect effectively not only against \textit{B. pertussis} but also against \textit{B. parapertussis}. We demonstrated previously that reciprocal protection is induced in mice infected with \textit{B. pertussis}

![Image](http://iai.asm.org/)
fected with *B. pertussis* were protected against *B. pertussis*. Injection of spleen cells from mice infected with *B. parapertussis* protected recipients against *B. parapertussis*. However, we did not detect any reciprocal protection against *B. pertussis* and *B. parapertussis*. We postulated, in our previous report (20), that FHA-specific serum antibodies and cell-mediated immunity against FHA might be related to the reciprocal protection. However, these factors might play a minimal role in reciprocal immunity, as indicated by the results of the present study. It is now necessary to examine the protective effects of FHA-specific antiserum and the corresponding splenocytes by using FHA from *B. pertussis* and *B. parapertussis*.

The numbers of CFU in lungs of mice were influenced by incubation of the pathogens, prior to inhalation, with the bronchoalveolar washes of mice infected with *B. pertussis* or with *B. parapertussis*. For challenges by *B. pertussis* that had been incubated with the bronchoalveolar washes of mice infected with *B. pertussis* or with *B. parapertussis*, the numbers of CFU in lungs were lower than the numbers of CFU in the lungs of control mice. We detected a decrease in the number of CFU in mouse lungs after a challenge with *B. parapertussis* that had been incubated with the bronchoalveolar washes of mice infected with *B. pertussis* or with *B. parapertussis*. These data suggest that mucosal immune responses, which include secreted antibodies, might be involved in reciprocal protection against *B. pertussis* and *B. parapertussis*. The protective effect of the bronchoalveolar washes of Bordetella-infected mice was significant but, nonetheless, weak. There was a difference in the ratio of the number of bronchoalveolar washes and the number of live cells of Bordetella in the lungs of aerosol-challenged mice in this experiment. In our aerosol infection model, mice were infected with approximately $10^5$ CFU/mouse. In this case, the bronchoalveolar wash of one mouse was used and so the ratio of washes to bacterial number was 1 wash to $10^3$ CFU. However, for the incubation of Bordetella cells with bronchoalveolar washes, we incubated the bronchoalveolar washes from seven mice with approximately $5 \times 10^9$ CFU of live Bordetella cells. In this case, the ratio was 7 washes to $5 \times 10^9$ CFU (1 wash to $7 \times 10^8$ CFU). It was thought that the concentration of factors related to protection in the latter case might be about $7 \times 10^3$ times lower than that in the former case. We examined the levels of IgG and IgA antibodies against whole cells of *B. pertussis* and of *B. parapertussis*. We did not detect cross-reactions between the sera from mice infected with *B. pertussis* and sera from mice infected with *B. parapertussis*. However, partial cross-reactions between lung homogenates from mice infected with *B. pertussis* and those from mice infected with *B. parapertussis* were detected. It has been reported that secretory IgA antibodies, induced by natural infection, are effective in achieving cross-protection against infection with heterologous influenza virus (11). However, although serum IgG antibodies provided effective protection against influenza virus, the antibodies did not establish cross-protection between variants of the virus (3, 16). Secretory IgA, which is a dimer of IgA monomers, has multivalent binding sites. It is known that multivalent binding between an antibody and an antigen results in a considerable increase in stability, compared to monovalent binding. Such a phenomenon might be related to the cross-reaction of IgA against *B. pertussis* and *B. parapertussis* in our study. Some antigens on the surfaces of *B. pertussis* and *B. parapertussis* cells that cross-reacted with secretory IgA from mice infected with *B. pertussis* and from mice infected with *B. parapertussis* might be related to reciprocal protection against *B. pertussis* and *B. parapertussis*, perhaps via inhibition of the attachment and/or colonization of these bacteria. In a previous study, we detected FHA-specific IgA antibodies in the lungs of mice that had been infected with *B. pertussis* or *B. parapertussis* (20), and it has been reported that FHA is important for the attachment of Bordetella to host cells (18). FHA-specific IgA antibodies might be involved in reciprocal protection against *B. pertussis* and *B. parapertussis*.

Our data suggest that the induction of mucosal antibodies might be important for the induction of reciprocal protection against *B. pertussis* and *B. parapertussis*. The induction of mucosal antibodies by currently available commercial pertussis vaccines, which are administered by injection, is very limited (8, 12, 13, 15). Immunization via a mucosal route, such as intranasal, is effective for the induction of mucosal immunity. Studies of mucosal immunization for the induction of reciprocal protection against *B. pertussis* and *B. parapertussis* are under way in our laboratory.

**REFERENCES**


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