Clearance of \textit{Bordetella parapertussis} from the Lower Respiratory Tract Requires Humoral and Cellular Immunity

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\textit{Bordetella parapertussis} and \textit{Bordetella pertussis} are closely related species that cause whooping cough, an acute, immunizing disease. Their coexistence in the same host populations at the same time and vaccine studies showing that \textit{B. pertussis} vaccines have little effect on \textit{B. parapertussis} infection or disease suggest that the protective immunity induced by each does not efficiently cross protect against the other. Although the mechanisms of protective immunity to \textit{B. pertussis} have been well studied, those of \textit{B. parapertussis} have not. The present study explores the mechanism by which \textit{B. parapertussis} is cleared from the lower respiratory tract by anamnestic immunity. Serum antibodies are necessary and sufficient for elimination of this bacterium, and CD4\(^+\) T cells, complement, and neutrophils are required for serum antibody-mediated clearance. Mice lacking immunoglobulin A had no defect in their ability to control or clear infection. Interestingly, serum antibody-mediated clearance of \textit{B. parapertussis} did not require Fc receptors that are required for antibody-mediated clearance of \textit{B. pertussis}. Together these data support a model for the mechanism of protective immunity to \textit{B. parapertussis} that is similar but distinct from that of \textit{B. pertussis}.

The bordetellae are a group of gram-negative respiratory pathogens that are capable of infecting a wide range of hosts. Among them are three closely related species that have recently been shown to be capable of infecting a wide range of hosts. \textit{Bordetella bronchiseptica}, \textit{Bordetella pertussis}, and \textit{Bordetella parapertussis} are closely related species that cause whooping cough, an acute, immunizing disease. Their coexistence in the same host populations at the same time and vaccine studies showing that \textit{B. pertussis} vaccines have little effect on \textit{B. parapertussis} infection or disease suggest that the protective immunity induced by each does not efficiently cross protect against the other. Although the mechanisms of protective immunity to \textit{B. pertussis} have been well studied, those of \textit{B. parapertussis} have not. The present study explores the mechanism by which \textit{B. parapertussis} is cleared from the lower respiratory tract by anamnestic immunity. Serum antibodies are necessary and sufficient for elimination of this bacterium, and CD4\(^+\) T cells, complement, and neutrophils are required for serum antibody-mediated clearance. Mice lacking immunoglobulin A had no defect in their ability to control or clear infection. Interestingly, serum antibody-mediated clearance of \textit{B. parapertussis} did not require Fc receptors that are required for antibody-mediated clearance of \textit{B. pertussis}. Together these data support a model for the mechanism of protective immunity to \textit{B. parapertussis} that is similar but distinct from that of \textit{B. pertussis}.

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without gentamicin resistance has been described previously (14). Liquid culture bacteria were grown at 37°C overnight on a roller drum to mid-log phase in Stainer-Scholte broth.

**Animal experiments.** C57BL/6, RAG2<sup>−/−</sup>, μMT, TCRα<sup>−/−</sup>, C5<sup>−/−</sup>, and Cd11b<sup>−/−</sup> mice were obtained from Jackson Laboratory (Bar Harbor, Maine). FeCr2<sup>−/−</sup> γ-common<sup>−/−</sup> mice were obtained from Taconic Laboratories (Germantown, New York). C3<sup>−/−</sup> mice, backcrossed extensively onto a C57BL/6 background, have been described elsewhere and were a gift of Rick Wetsel (5). IgA<sup>−/−</sup> mice were a kind gift from Innocent Mbawuike (26). All mice were bred in a *Bordetella*-free environment. Mice were slightly sedated with isofluorane (Abbott Laboratories) and inoculated by pipetting 50 μl of phosphate-buffered saline (PBS) containing approximately 5 × 10<sup>6</sup> *B. parapertussis* onto the tip of the external nares as previously described (22). For time course experiments, groups of three or four animals were sacrificed on days 3, 7, 14, 28, 49, 70, and/or 105 postinoculation. Lung and systemic colonization was quantified by homogenizing tissues in PBS, plating onto Bordet-Gengou blood agar containing 20 μg/ml gentamicin, and counting the number of colonies. Statistical analysis was performed using the Student t test when comparing numbers of CFU. Values yielding P values less than 0.05 were considered statistically significant. Adoptive transfer experiments were conducted by injecting 200 μl of convalescent-phase serum intraperitoneally (i.p.) at the time of inoculation. Convalescent-phase serum samples were collected from wild-type mice infected with *B. parapertussis* 28 days postinoculation. For the production of survival curves, once the progression of disease was clear, moribund animals were euthanized to prevent unnecessary stress. All animals were handled in accordance with institutional guidelines.

**Cell depletions.** PMN depletion was done by injecting 1 mg of the monoclonal Ab from the RB6-8C5 hybridoma i.p. (8, 9) 24 hours prior to and on days 3, 7, 14, 28, 49, 70, and/or 105 after infection. CD4<sup>+</sup> T cells were depleted by injections of 1 mg of the monoclonal Ab from the GK1.5 hybridoma at days 0 and 7. Similarly, CD8<sup>+</sup> T cells were depleted by injection of 1 mg of the monoclonal Ab from the YTS168-4 hybridoma at days 0 and 7 (31).

**Enzyme-linked immunosorbent assays (ELISAs).** Bacteria were grown overnight to an optical density at 600 nm of 0.7, heat inactivated, diluted in carbonate buffer, and used to coat the wells of 96-well plates. Plates were stored at 4°C (wells filled with PBS containing Tween 20 [PBS-T] plus 1% bovine serum albumin) until use. A 1:50 dilution of convalescent-phase serum samples from different mouse strains was added to the first wells and serially diluted 1:2 across the plates. The plates were incubated for 2 h at 37°C in a humidified chamber and then washed three times with PBS-T. Polyvalent anti-mouse secondary Abs were used to look at the total titer (1), and specific isotypes were determined by using the appropriate secondary Abs (Southern Biotechnology Associates and Pharmingen). The plates were then incubated at 37°C in a humidified chamber for 1 h before they were washed four times with PBS-T. 2.2′-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) in a phospho-citrate buffer and hydrogen peroxide were added to the wells, which were then incubated at room temperature in the dark for 30 min. A sodium fluoride solution was added to the wells to stop the reaction, and the plates were read at an absorbance of 405 nm.

**RESULTS**

An adaptive immune response is required to survive infection by *B. parapertussis*. In both humans and mice, *B. parapertussis* causes an acute infection of the LRT. CFU in the lungs have been shown to increase rapidly more than 100-fold during the first week of infection but decline thereafter in wild-type mice (22). In SCID-beige mice, which are deficient in B, T, and natural killer cells, this decline is not observed and the infection is lethal (14, 19), indicating that these cells are necessary to control *B. parapertussis*. In order to identify which aspects of the adaptive immune response are required for control and clearance, different immunodeficient strains of mice were infected with *B. parapertussis*. Groups of 10 C57BL/6 (wild-type), RAG2<sup>−/−</sup> (B- and T-cell-deficient), μMT (B-cell-deficient), and TCR-α<sup>−/−</sup> (αβ-cell-deficient) mice were given an intranasal inoculation of 5 × 10<sup>6</sup> CFU of *B. parapertussis* in 50 μl PBS. Wild-type, μMT, and TCR-α<sup>−/−</sup> mice survived 105 days postinoculation without any signs of distress and were then euthanized. On day 17 postinoculation, RAG2<sup>−/−</sup> mice began to show symptoms of disease, such as hunched posture, decreased level of activity, and ruffled fur. These mice began dying on day 18, and all were dead by day 28 (Fig. 1A). In a subsequent experiment, *B. parapertussis* numbers in the LRT and blood were measured for the same mouse strains on day 28 postinoculation. Wild-type C57BL/6 mice reduced *B. parapertussis* to about 100 CFU in the LRT by day 28. However, RAG2<sup>−/−</sup>, μMT, and TCR-α<sup>−/−</sup> mice were all unable to reduce bacterial numbers below the maximal levels observed on days 5 and 7 in wild-type mice (∼10<sup>6</sup> to 10<sup>7</sup> CFU). Although each of these immunodeficient mouse strains was unable to efficiently reduce colonization in the lungs (Fig. 1B), Rag2<sup>−/−</sup> mice were the only strain that allowed colonization of the blood by *B. parapertussis* (Fig. 1C). The observation that only mice lacking both B cells and T cells succumb to lethal, systemic *B. parapertussis* infection suggests that either B cells or T cells can control the spread from the respiratory tract.
Mice deficient in Ab production are unable to clear *B. parapertussis* from the LRT. To assess the role of adaptive immunity in the elimination of *B. parapertussis* from the LRT, the level of colonization was monitored in C57BL/6, RAG2<sup>−/−</sup>, μMT, and TCR-α<sup>−/−</sup> mice for 105 days to determine whether they were able to clear the infection. Groups of 24 mice were infected, and 4 mice of each strain were sacrificed on days 7, 14, 28, 49, 70, and 105. C57BL/6 mice reduced *B. parapertussis* to low numbers in the LRT by day 28, and no bacteria could be recovered by day 49 postinoculation. RAG2<sup>−/−</sup>, μMT, and TCR-α<sup>−/−</sup> mice were all defective in bacterial clearance compared to wild-type mice, and high numbers of *B. parapertussis* were still present in the LRT at 105 days postinoculation (Fig. 2A). Serum samples were taken from mice sacrificed on day 28 postinoculation to quantify the titers of anti-*B. parapertussis* polyclonal Abs, immunoglobulin A (IgA), IgG, IgG1, IgG2a, IgG2b, IgG3, and IgM by ELISAs. Anti-*B. parapertussis* IgA and IgG3 were undetectable in the sera of any infected mice (data not shown). While C57BL/6 mice produced high titers of anti-*B. parapertussis* Abs of other isotypes, μMT and TCR-α<sup>−/−</sup> mice were defective in the production of all isotypes except for the production of IgM by TCR-α<sup>−/−</sup> mice (Fig. 2B).

Mouse strains that were unable to produce anti-*B. parapertussis* Abs were also defective in the clearance of the bacteria from the LRT, suggesting that Abs may be critical to the elimination of *B. parapertussis* from the LRT.

**Serum Abs are required to clear *B. parapertussis* from the LRT, while mucosal Abs are not.** Since *B. parapertussis* is an extracellular mucosal pathogen, we examined the role of IgA Abs, which is the predominant isotype within mucosal secretions. Twenty-four IgA<sup>−/−</sup> mice were inoculated with *B. parapertussis*, and groups of 4 were sacrificed 7, 14, 28, 49, 70, and 105 days later to monitor the colonization level in the LRT. While mice that were ineffective in producing *B. parapertussis*-specific Abs were unable to clear bacteria from the LRT, mice lacking only IgA eliminated the bacteria with kinetics similar to that of wild-type mice (Fig. 3). This finding indicates that IgA is not required and suggests that other isotypes are sufficient to clear *B. parapertussis* from the LRT.

**Adoptive transfer of serum Abs clears *B. parapertussis* from the LRT but requires help from T cells.** An adoptive transfer model was used to determine whether serum Abs are sufficient to eliminate *B. parapertussis*. Groups of four mice were injected i.p. with 200 μl of naïve or immune sera (collected from wild-type mice 28 days postinoculation) and inoculated with *B. parapertussis* (as described above) on day 0 and sacrificed on day 14. Although previous studies showed that transferring immune serum to naïve wild-type mice upon infection has no effect on bacterial numbers in the first 7 days postinoculation (22), we observed a substantial effect thereafter. C57BL/6 mice given naïve serum had colonization levels similar to those of untreated C57BL/6 mice (~10<sup>5</sup> CFU) (Fig. 4). In contrast, both C57BL/6 and μMT mice treated with immune serum lowered bacterial numbers to less than 100 CFU in the LRT by...
Both mouse strains were able to significantly decrease numbers of *B. parapertussis* by day 14 postinoculation (approximately 99% clearance), although they did not completely clear the infection (Fig. 5). These results suggest that Fcγ receptors are not required for efficient reduction of *B. parapertussis* numbers, although they may contribute to clearance. Together these data indicate that only complement factors upstream of C5 activation are required for the efficient function of serum Abs against *B. parapertussis*.

**PMNs are required for the Ab-mediated clearance of *B. parapertussis***. The fact that antibody-mediated clearance of *B. parapertussis* requires C3, but not C5, suggests that elimination occurs via phagocytosis of C3b- and Ab-coated bacteria. Since we have recently noticed that PMNs are crucial to controlling *B. pertussis* and *B. bronchiseptica* infections (23; unpublished data), the role of PMNs in the Ab-mediated clearance of *B. parapertussis* was tested by depleting these cells with an i.p. injection of the monoclonal Ab RB6-8C5 24 h prior to and 7 days after infection (8, 9). Groups of four μMT mice were given i.p. injections of RB6-8C5 or left untreated and then injected with immune serum and inoculated with *B. parapertussis*. Mice receiving immune serum alone completely cleared infection by day 14 postinoculation. However, in mice depleted of PMNs, immune serum had no effect on *B. parapertussis* numbers, which increased to greater than 10^6 CFU in the lungs by day 14 (Fig. 6). These data indicate that PMNs are required for serum Abs to eliminate *B. parapertussis* from the LRT.

**DISCUSSION**

Our results show that Abs are crucial for the elimination of *B. parapertussis* from the LRT. Interestingly, serum Abs cleared this extracellular mucosal pathogen from the LRT, while IgA was not essential. Our study of serum Ab-mediated...
clearance of *B. parapertussis* suggests that Ab-opsonized bacteria activate the complement cascade, become coated by C3b, and are subsequently phagocytosed by PMNs via Fcγ receptors and/or CR3. In this model, either Fcγ receptors or CR3 are sufficient for reduction of the bacterial load (about 99% clearance), but the presence of both receptors results in complete elimination of bacteria. Furthermore, our data also suggest that helper T cells, but not cytotoxic T cells, are necessary for the function of Abs. Although the exact role of helper T cells in facilitating the bacterial clearance is not known, our preliminary studies suggest that they aid in the recruitment and perhaps activation of PMNs in the lungs via gamma interferon production (unpublished data). It appears that both humoral and cellular responses are crucial in immunity to *B. parapertussis*.

Recent clinical surveys indicate that *B. parapertussis* may be responsible for a large percentage of diagnosed cases of whooping cough (40). Although the immune response to *B. pertussis* is fairly well characterized, the immune response to *B. parapertussis* has largely been ignored. Our data show that like *B. pertussis*, both humoral and cellular immune responses are required for sterilizing immunity to *B. parapertussis*. Since these two species are closely related, it would be expected that protective immunity induced by either one would induce cross-protection against the other. In fact, experimental studies have shown that these two species induce a limited level of cross-protection, clearly not as high as their levels of self-protection (10, 18, 21, 41). This incomplete cross-protection could result from the two species being antigenically distinct in each of their many surface and secreted proteins. Alternatively, a different set of prominent surface molecules could also prevent effective immune cross-protection. O antigen is the dominant surface antigen of *B. parapertussis* but is absent from *B. pertussis* isolates. Our previous work has shown that O antigen is required for efficient colonization of the murine respiratory tract by *B. parapertussis* (3), but its role in eliciting a protective immune response or conversely, protecting bacteria from the adaptive immune response is largely unknown. O antigen is expressed by several different bacteria and has various functions in vivo and in vitro, including contributing to host colonization, resistance to complement-mediated killing, and the ability to cause sepsis (3, 6, 27, 34, 35). Our previous studies have shown that the O antigens of *B. parapertussis* and *B. bronchiseptica* protects them from killing by complement in the absence of Abs, while *B. pertussis*, which lacks this virulence factor, is naturally sensitive to the effects of naïve serum complement (3). It is unknown at this time whether O antigen has other functions in *B. parapertussis* infections in addition to conferring resistance to complement-mediated killing. As a major component of the outer surface of the bacterium, it is possible that it either shields other protective antigens or presents a decoy antigen. The lack of expression of pertussis toxin (ptx) by *B. parapertussis* is another key difference between this bacterium and *B. pertussis*. Compared to wild-type *B. pertussis* strains, strains deleted of ptx are not able to colonize the respiratory tract as efficiently (4). However, *B. parapertussis* is able to reach high levels in the LRT without this toxin, ptx is a prominent antigen of *B. pertussis* to which high titers of specific Abs are produced (38). Thus, it is possible that by not expressing this toxin, *B. parapertussis* may avoid *B. pertussis*-induced immunity.

Ecological theory holds that two antigenically similar pathogens cannot occupy the same host population indefinitely due to immune-system-mediated competition; the more virulent of the two will have the short-term advantage of increased transmission but is more likely to undergo epidemic fadeout (13). The coexistence of *B. parapertussis* and *B. pertussis* in the same host population, and occasionally in the same individual (16), indicates that these organisms have some mechanism to avoid immune-system-mediated competition. Phylogenetic analysis suggests that *B. parapertussis* emerged from a *B. bronchiseptica*-like progenitor and adapted to humans more recently than *B. pertussis* did (29). Thus, it could be predicted that *B. parapertussis* successfully invaded a human population in which *B. pertussis* was already prevalent and, consequently, needed to develop mechanisms to avoid *B. pertussis*-induced immune responses. The presence of *B. parapertussis* and *B. pertussis* in the same population is intriguing, and understanding their abilities to coexist could provide an excellent model to study evolution, adaptation, and spread in the emergence of bacterial pathogens.

While whooping cough has recently been increasing in prevalence in vaccinated populations, the dangers of *B. parapertussis* and *B. pertussis* infections lie in the spread of these bacteria to unvaccinated individuals or those in whom immunity has waned. Both species are quite capable of colonizing *B. pertussis*-vaccinated individuals, often going undiagnosed (2, 16, 40), and whooping cough epidemics are frequent and periodic in some populations (15, 33). Control of these pathogens at the population level requires greater understanding of immune mechanisms that could provide protection not only against severe disease but also against subclinical infections. Such understanding would allow for the design of novel vaccine strategies that induce robust and long-lasting humoral and cellular immune responses against both *B. parapertussis* and *B. pertussis*. 

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**FIG. 6.** Neutrophils are required for the function of serum antibodies against *B. parapertussis*. Four control µMT mice were injected i.p. with naïve serum (NS) upon inoculation as described in the legend to Fig. 1. Four µMT mice were injected i.p. with immune serum (IS) upon inoculation. Four µMT mice were injected with the monoclonal antibody RB6-8C5 (RB6) to deplete neutrophils 24 h prior to and 7 days after infection and injected with immune serum upon inoculation. All mice were sacrificed on day 14. The numbers of bacteria are expressed as the log10 means ± standard deviations (error bars). Values that were significantly different from those for wild-type C57BL/6 mice (P values of less than 0.05) are indicated by asterisks. The dashed line represents the limit of detection.
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