ANTIBODY-MEDIATED CLEARANCE OF

*BORDETELLA* SPECIES

A Thesis in
Pathobiology

by
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ABSTRACT

This doctoral dissertation work focused on examining the contribution of mucosal and serum antibodies to sterilizing immunity against the respiratory pathogens, *B. bronchiseptica* and *B. pertussis*. During the course of the investigation, we discovered that the mechanism of antibody-mediated clearance of these closely related subspecies of *Bordetella* differ significantly. Adoptively transferred serum antibodies cleared the animal pathogen *B. bronchiseptica* within three days of infection. Antibodies, however, had a minimal effect on human adapted *B. pertussis* until after the generation of a Th1 response. Comparative immunobiology of these two subspecies in a mouse model revealed that *B. pertussis* is initially able to resist antibody-mediated clearance via the expression of pertussis toxin, which modulates the critical immune responses required for this rapid bacterial elimination. This suggests a model in which *B. pertussis* is able to transiently infect immune individuals and persist in vaccinated populations. This model is supported by epidemiological data indicating frequent epidemics of whooping coughs in highly vaccinated populations. Additionally, this study revealed distinct yet overlapping immune surveillance mechanisms in different areas of the respiratory tract. Together, this study provides important insights into the evolution, adaptation, and epidemiology of *B. pertussis*. It also aids in developing novel prophylactic and therapeutic strategies for whooping cough. Above all, it makes substantial contributions to the respiratory immunology.
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CHAPTER 1

INTRODUCTION: *BORDETELLA*
Members of the genus *Bordetella* are minute gram-negative cocobacilli that cause primary and opportunistic infections in a wide range of animals including reptiles, birds, and mammals (28, 33). The genus consists of 8 members of which *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, sometimes referred as classical *Bordetella*, are best studied (9). *B. pertussis* causes the human disease, whooping cough, primarily in young children (5). *B. parapertussis* causes a similar disease but the incidence of *B. parapertussis* infection is underestimated (14). In fact several surveys indicate that between 5 and 25% of pertussis cases may be caused by this pathogen (20). *B. bronchiseptica* causes a wide spectrum of disease ranging from acute (kennel cough in dogs and atrophic rhinitis in piglets) to very mild (suffles in rabbits) (15, 36). Among non-classical bordetellae, *B. avium*, the causative agent of turkey coryza, is best studied and is isolated from a variety of birds (41). Other bordetellae, such as *B. hinzii*, *B. homseii*, and *B. trematum*, have been occasionally isolated from immunocompromised humans (6, 42, 45, and 46).

**Evolution of Bordetella**

*B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are considered to be extremely closely related based on multilocus enzyme electrophoresis, insertion sequence typing, and DNA hybridization studies (26, 44). Recently, *B. pertussis* and *B. parapertussis* have been reclassified as subspecies of *B. bronchiseptica* (26). It is believed that two independent events led to the evolution of the human-adapted subspecies, with *B. pertussis* and *B. parapertussis* arising from different lineages and being more closely related to *B. bronchiseptica* than to each other (Fig. 1) (44). However, *B. pertussis* and *B. parapertussis* commonly infect only humans while *B. bronchiseptica*
continues to infect a variety of mammals (33). Recent sequencing of the genomes of all three subspecies revealed that *B. bronchiseptica* has almost all the genes that are present in the other two subspecies (29). However, the size of the genomes of *B. pertussis* and *B. parapertussis* is significantly smaller than that of *B. bronchiseptica* (approximately 23.5% and 10.5% of total genome, respectively) (Table 1) (29). Thus the adaptation of *B. pertussis* and *B. parapertussis* to humans correlates with a loss of genes rather than gain of genes (33). The close relatedness yet distinct biology of these three subspecies together with a common mouse model provide an excellent opportunity to study molecular aspects that may have lead to their differential host adaptation and pathogenesis.

**Pathogenesis**

All three subspecies have a two-component system BvgAS that regulates expression of various virulence factors, defined broadly as factors that contribute to transmission, infection, and pathogenesis (8). Although, the exact physiological signals for BvgAS are not known, temperature, nicotinic acid and sulfate ions modulate the BvgAS system in vitro (8). Genes that are expressed under Bvg⁺ condition (37°C) are necessary and sufficient for virulence of bordetellae (19). The functions of Bvg⁻ phase genes are not known but thought to allow bacteria, particularly *B. bronchiseptica*, to survive in the environment between hosts. Recently, an intermediate Bvg phase, Bvg⁺, has been described but the function of this phase is not known (7). Various well characterized Bvg⁺ genes and their functions are listed in Table 2.

Virulence factors of special interest to this study include Bvg regulated pertussis toxin (PTX) and lipopolysaccharide (LPS). *B. pertussis* is the only species of *Bordetella* that expresses PTX (30). Interestingly, *B. bronchiseptica* does not express PTX but
maintains an intact operon for synthesis and secretion of PTX (29). Even though B. *parapertussis* is adapted to humans and produces a similar disease to that of *B. pertussis*, it does not express PTX(29). PTX has been extensively studied in vitro for its inhibitory effects on G-protein coupled receptor signaling and adjuvant properties (25), but its in vivo role in *B. pertussis* pathogenesis had not been described prior to the studies described here.

These three subspecies have distinct LPS structures: that of *B. pertussis* resolves into two bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and are termed band A and B (13). Band B is composed of lipid A and a branched-chain core oligosaccharide. Band A is composed of a trisaccharide attached to band B. *B. bronchiseptica* expresses LPS molecules that are very similar electrophoretically to *B. pertussis* bands A and B, but also contain an O-antigen-like homopolymer of 2,3-dideoxy-2,3-di-N-acetylgalactosaminuronic acid (2,3-di-NAcGalA)(13). *B. parapertussis* expresses a faster-migrating minimal molecule (band B'), as well as a large molecule containing the same O-antigen-like structure as *B. bronchiseptica*, and does not express the trisaccharide(13). *B. bronchiseptica* and *B. parapertussis*, in addition to O-antigen, have a hexa-acylated lipid A structure, whereas *B. pertussis* LPS is penta-acylated (32). The relative importance of each of these structures in pathogenesis and immunity has been examined but is not yet fully understood (4, 13, 31, and 32).

**Immunity**

Immunity to *B. pertussis* infection has been studied in an effort to improve vaccines to whooping cough and as such vaccine-induced immunity is well elucidated.
The majority of early *B. pertussis* vaccine studies focused primarily on protection from disease. Accordingly, an infant mouse model and a mouse intracerebral challenge model of pertussis have been used extensively (35, 50). However, growing evidence for the resurgence of whooping cough in both children and adults, as well as the presence of subclinical infections in the immune population (49) validates the necessity of studying immunity against colonization/infection. A respiratory challenge model in adult mice was thus developed in the early 1990s (23), and has been extensively used to measure the efficacy of vaccines and to understand basic immunity against *B. pertussis* (11, 2). Most reports, however, consider marginal reduction in the numbers of *B. pertussis* recovered from the respiratory tract even after 2 weeks post-challenge as significant (22). Unfortunately, this prolonged shedding period allows transmission of *B. pertussis* to susceptible individuals. A more efficient vaccine would protect against colonization, thereby minimizing transmission, and providing population immunity.

The pertussis whole cell vaccine (Pw), is prepared from whole killed bacteria (11), and has been extensively used to limit pertussis in both developed and developing nations (2). Safety concerns following adverse reactions to whole cell vaccines prompted the switch to pertussis acellular vaccines (Pa) in developed countries (21). These vaccines have one or more inactivated *B. pertussis* virulence components such as pertussis toxin (PTX), hemagglutinin (HA), pertactin (PTA), and fimbriae (Fim) (12, 40). Acellular vaccines are costly to produce, and consequently have seen limited use in underdeveloped countries, which continue to use Pw (48). Although Pa has reduced the incidence of clinical disease, recent data highlighting adverse reactions and apparent
short term immunity, has raised questions of the safety and efficacy of these vaccines as well (16).

The majority of research dealing with vaccines to *B. pertussis* has focused either on cellular or humoral immunity. Yet both cell-mediated and antibody-mediated immune responses are now thought to be important for protection. In a murine model of pertussis, both B cells and T cells are required for infection-induced, as well as vaccine-induced immunity (17, 18). Specific roles for each cell type in protection have not been defined. It is thought that while B cells are required for functions other than antibody production, T cells may be required for production of cytokines (17, 18). There are no reported studies that consider the interaction between cell-mediated and humoral immune responses, and seek to explain how they complement each other in the overall response to *B. pertussis*.

Infection-induced immunity to *B. pertussis* is strongly biased towards a Th1 type T cell response, both in humans and in mice (3, 18). Pw has also been shown to induce a Th1 type response (3). Alternatively, Pa induces a robust Th2 type response and is less protective than Pw (37). This has prompted investigators to suggest that a Th1 type response is required for complete protection against pertussis (23). These thoughts were further supported by findings that a Th1 type is associated with protection in both humans and mice (34), but the specific mechanism by which a Th1 type immune response protects against extracellular pathogens that reside in the lumen of airways is not known.

B cell deficient mice are unable to clear *B. pertussis* from the respiratory tract, suggesting antibodies may have a role in the clearance of *B. pertussis* (17, 18). Both previously infected and immunized humans show a significant increase in serum
antibody titers against various *B. pertussis* antigens (10, 12, 27, 40, and 43). In the mouse model of *B. pertussis*, infected and immunized mice show antibody titer patterns similar to those of humans (24, 39). However, it has previously been reported that adoptive transfer of *B. pertussis* convalescent serum to infected mice had little effect on bacterial numbers in the lungs up to 7 days post-infection (18). Conflicting reports regarding the role of antibodies in prevention and protection from infection necessitate a detailed understanding of the mode antibody action and their dependence on other cellular immune responses. In spite of their importance in public and animal health, very little is known about immunity to *B. parapertussis* and *B. bronchiseptica*. We hypothesize that **antibodies are required for eliminating *B. pertussis*, *B. parapertussis** and *B. bronchiseptica from the respiratory tract** and utilize a common mouse model to determine the role of antibodies in bacterial clearance in the respiratory tract.
Fig. 1 Phylogenetic tree of *Bordetella*. A) 16s rDNA-based phylogenetic tree of type strains of the family Alcaligenaceae. B) Schematic presentation of phylogenetic relationships within the *B. bronchiseptica* cluster based on the distribution of insertion elements and MLEE. Adapted from Gelrach G et al, 2001, Microbes and Infection, 3, 61-72

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<td>+</td>
<td>- (Pseudogenes)</td>
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<td>- (Only a few strains have genes)</td>
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Table 2. Virulence factors of *Bordetella* and their distribution and functions
REFERENCES

1. Barnard, A., B. P. Mahon, J. Watkins, K. Redhead, and K. H. Mills. 1996. Th1/Th2 cell dichotomy in acquired immunity to Bordetella pertussis: variables in the in vivo priming and in vitro cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0. Immunology 87:372-80.


CHAPTER 2
ROLE OF ANTIBODIES IN IMMUNITY TO *BORDETELLA*
INFECTIONS
ABSTRACT
The persistence of *Bordetella pertussis* and *B. parapertussis* within vaccinated populations and the reemergence of associated disease highlight the need to better understand protective immunity. The present study examined host immunity to bordetellae and addressed potential concerns about the mouse model by using a comparative approach including the closely related mouse pathogen *B. bronchiseptica*. As previously observed with *B. pertussis*, all three organisms persisted throughout the respiratory tracts of B-cell-deficient mice, indicating that B cells are required for bacterial clearance. However, adoptively transferred antibodies rapidly cleared *B. bronchiseptica* but not human pathogens. These results obtained with the mouse model are consistent with human clinical observations, including the lack of correlation between antibody titers and protection, as well as the limited efficacy of intravenous immunoglobulin treatments against human disease. Together, this evidence suggests that the mouse model accurately reflects substantial differences between immunities to these organisms. Although both *B. pertussis* and *B. parapertussis* are more closely related to *B. bronchiseptica* than they are to each other, they share the ability to resist rapid clearance from the lower respiratory tract by adoptively transferred antibodies, an adaptation that correlates with their emergence as human pathogens that circulate within vaccinated populations.
INTRODUCTION

*Bordetella bronchiseptica*, *B. pertussis*, and *B. parapertussis* are closely related gram-negative respiratory pathogens that have recently been reclassified as subspecies (12,16). *B. pertussis* and *B. parapertussis* appear to have diverged independently from a *B. bronchiseptica*-like progenitor and are highly infectious pathogens that primarily infect humans, causing the acute and severe disease pertussis or whooping cough (5, 6). In contrast, *B. bronchiseptica* infects a wide range of mammals (4), typically asymptomatically, and persists in the upper respiratory tract indefinitely (4). The basis for the interspecies differences in host range and severity of disease is not known, but these differences may be related to differences between bacterial subspecies or host differences in physiology or immune response to *Bordetella* infection.

Little is known definitively about the normal human immune response to *Bordetella* infection because it has generally been studied in individuals who were previously vaccinated (10). In the murine model, B cells are necessary to eliminate *B. pertussis*, suggesting that antibodies have a critical role in clearance (9). Although the importance of antibodies in immunity to other bacterial respiratory pathogens, such as *Haemophilus influenzae* and *Pasteurella multocida*, are well documented (10) and *Bordetella*-specific antibodies are generated in response to vaccination or infection (15), anti-*Bordetella* titers do not correlate well with protection in large clinical trials (3). In contrast to natural immunity following an infection, vaccination provides little, if any, protection against subclinical infection (10) and does not protect from cross infection with other *Bordetella* subspecies despite generating a strong antibody response (15). Understanding natural immunity to bordetellae may allow the design of better vaccines.
that not only reduce the severity of the disease but also prevent infection and provide cross protection against other bordetellae.

In order to investigate the comparative biology of these closely related organisms, we have examined the basis for protective immunity to each in the mouse model. Experiments with SCID and RAG1−/− mice indicated that adaptive immunity is required to clear all three organisms from the lower respiratory tract (4). B-cell-deficient mice fail to clear B. pertussis suggesting that antibodies may have a role in clearance of B. pertussis (9), but the role of antibodies in immunity to B. bronchiseptica and B. parapertussis is not known. Here we demonstrate that serum antibodies completely clear B. bronchiseptica from the lower respiratory tracts of wild-type and B-cell-deficient mice within 3 days but have no effect on the human-adapted pathogens in this time frame. This interspecies difference could not be attributed to antibody titers or differences in serum isotypes. We discuss the possibility that the human pathogens acquired resistance to serum antibodies during their apparently independent evolution from B. bronchiseptica-like animal pathogens in order to persist in immune populations.
MATERIALS AND METHODS

**Bacteria.** Bacteria were maintained on Bordet-Gengou agar (Difco), inoculated into Stainer-Scholte broth at optical densities of 0.1 or lower, and grown to mid-log phase at 37°C on a roller drum. Wild-type strains of *B. bronchiseptica* (RB50), *B. parapertussis* (12822), and *B. pertussis* (BP536) have been described previously (4,5).

**Animal experiments.** C57BL/6 and MuMT mice were obtained from The Jackson Laboratory. Mice lightly sedated with isoflurane (Abbott Laboratories) were inoculated by pipetting 50 µl of phosphate-buffered saline (PBS) containing 5 x 10⁵ bacteria onto the tip of the external nares. For time course experiments, groups of four animals were sacrificed on days 0, 3, 7, 14, 28, 49, 70, and 105 postinoculation. Colonization of various organs was quantified by homogenization of each tissue in PBS, plating onto Bordet-Gengou blood agar containing 20 µg of streptomycin per ml, and colony counting. For passive-transfer experiments, wild-type mice were inoculated with 5 x 10⁵ CFU of *B. bronchiseptica*, *B. parapertussis*, or *B. pertussis* by the intranasal route as described above and serum was collected on day 28 postinoculation. Two hundred microliters of convalescent-phase or naive serum was injected intraperitoneally into mice before inoculation. Animals were sacrificed on days 0, 1, 3, 5, and 7 postinoculation or as indicated in each experiment. Colonization of various organs was quantified as described above. All animal experiments were carried out in accordance with institutional guidelines. Statistical significance of data points was determined using a student's unpaired t-test.
**Antibodies.** Titers of anti-*Bordetella* antibody in convalescent-phase sera were determined by enzyme-linked immunosorbent assay with polyvalent anti-mouse secondary antibodies as described previously (1). Specific classes and isotypes of antibodies were determined by using appropriate secondary antibodies (Southern Biotech, Birmingham, AL and Pharmingen, San Diego, CA).
RESULTS

**B cells are necessary for the clearance of bordetellae from the respiratory tracts of mice.** The role of B cells in immunity against bordetellae was investigated by using B-cell-deficient MuMT mice (7). Wild-type and MuMT mice were inoculated intranasally with $5 \times 10^5$ CFU of various *Bordetella* subspecies in 50 µl of PBS. This inoculation regimen consistently delivers approximately $10^5$ CFU to the nasal cavity and lungs and $10^3$ CFU to the trachea (4). Bacterial numbers were determined in the nasal cavity, trachea, and lungs at various time points. In wild-type mice, bacterial numbers began to decrease after day 7 and *B. bronchiseptica* and *B. parapertussis* were cleared from the lower respiratory tract (trachea and lungs) by day 70 while *B. pertussis* was cleared by day 49 postinoculation (Fig. 1). In contrast, MuMT mice failed to clear the three *Bordetella* subspecies from the lower respiratory tract even on day 105 post-inoculation and bacterial numbers were comparable to those recovered on day 7. These results agree with data published earlier showing that *B. pertussis* persists in MuMT mice (9) and indicate that B cells are required for the decrease in bacterial numbers observed after day 7 in wild-type mice.

MuMT mice were also defective in controlling bordetellae in the nasal cavity. Although *B. bronchiseptica* and *B. parapertussis* normally persist in the nasal cavities of wild-type mice beyond 105 days postinoculation, bacterial numbers were significantly (10²- to 10⁴-fold) higher in this site in MuMT mice than in wild-type mice (Fig. 1A and B). *B. pertussis* was cleared from wild-type mice by day 49 but was recovered from the nasal cavities of MuMT mice until at least day 105 in numbers similar to those of *B. bronchiseptica* and *B. parapertussis* (Fig. 1). These data indicate that B cells are required
for the >100-fold reduction in the numbers of *B. bronchiseptica* and *B. parapertussis* and for the elimination of *B. pertussis* from the nasal cavities of wild-type mice.

**Serum antibodies are sufficient to clear *B. bronchiseptica*, but not *B. parapertussis* or *B. pertussis*, from the lower respiratory tracts of mice.** Although B cells appear to be required for the clearance of all three *Bordetella* subspecies, previous studies have shown that antibodies have little effect on *B. pertussis* numbers in vivo, leading to the conclusion that B cells are required for some function other than antibody production (8). To examine the specific role of antibodies in the control and clearance of bordetellae, serum from naive or convalescent animals previously infected with each of the *Bordetella* subspecies was adoptively transferred into naive animals immediately prior to a challenge with the respective subspecies. *B. bronchiseptica*-induced convalescent-phase serum (serum collected from mice 28 days postinoculation with *B. bronchiseptica*) rapidly cleared *B. bronchiseptica* from the tracheas and lungs of mice by day 3 postinoculation, whereas naive serum had no significant effect (Fig. 2A). In striking contrast, both human pathogens were unaffected by adoptive transfer of respective convalescent-phase (Fig. 2B and C). Interestingly, adoptive transfer of convalescent-phase serum had no significant effect on the number of bacteria recovered from the nasal cavity. These data reveal both tissue-specific and bacterium-specific effects of antibodies and, together with the data presented in Fig. 1, indicate that some factor that is missing from B-cell-deficient mice and is not replaced by transfer of serum antibodies is required for clearance of the human pathogens from the lower respiratory tract and for a greater-than-100-fold reduction in the numbers of all three bacterial subspecies in the nasal cavity.
To further investigate the possibility that B cells are required for some function other than antibody production, naive or convalescent-phase serum was transferred into B-cell-deficient MuMT mice challenged with *B. bronchiseptica*. As observed in wild-type animals, naive serum had no effect on bacterial numbers but convalescent-phase serum rapidly (by day 3) eliminated *B. bronchiseptica* from the lower respiratory tracts of these B-cell-deficient mice (Fig. 3). These results suggest that no additional B-cell functions are required for efficient serum antibody-mediated clearance of *B. bronchiseptica* from the lower respiratory tract. Additionally, serum from MuMT mice infected with *B. bronchiseptica* and collected on day 28 postinoculation was adoptively transferred into mice infected with *B. bronchiseptica*. Unlike wild-type serum, serum from MuMT mice had no effect on *B. bronchiseptica* numbers in the lower respiratory tracts of mice (data not shown). These data suggest that antibodies are responsible for the rapid clearance of *B. bronchiseptica* by convalescent-phase serum.

**Antibody titers and clearance.** We hypothesized that the differences in the effectiveness of serum antibodies in clearing the three *Bordetella* subspecies from the lower respiratory tracts of mice could be due to quantitative and qualitative differences in the antibodies present in the respective convalescent-phase sera. We therefore determined the anti-*Bordetella* antibody titers of various classes and isotypes of antibodies in these sera. There was no significant difference in the overall titers or individual isotypes of anti-*Bordetella* antibodies generated by *B. bronchiseptica* or *B. parapertussis*, but both induced significantly higher antibody titers than did *B. pertussis* (Fig. 4). To investigate whether the lower antibody titer of *B. pertussis*-induced serum could be involved in the lack of antibody-mediated clearance in vivo, mice were administered three times the
volume of *B. pertussis*-induced serum. Even when antibody titers were compensated for in this way, *B. pertussis*-induced serum had no effect on bacterial numbers within the respiratory tract (Fig. 5). To investigate the possibility that some qualitative characteristic of the antibodies was involved in their differential activity, *B. bronchiseptica*-induced serum was transferred into animals infected with *B. pertussis*. Although this serum recognized and bound *B. pertussis* in vitro as well as it did *B. bronchiseptica* as determined by western blot analysis, it did not affect *B. pertussis* numbers in the respiratory tracts of mice (data not shown). These data suggest that the difference in the susceptibility of human-adapted bordetellae to serum antibodies is not due to quantitative differences in the antibodies. To investigate the possibility of the presence of some inhibitors in *B. pertussis*-induced serum interfering with the action of antibodies, we adoptively transferred both *B. pertussis*- and *B. bronchiseptica*-induced sera into mice infected with *B. bronchiseptica*. These mice efficiently cleared *B. bronchiseptica* from the trachea and lungs within 3 days post-treatment (data not shown). These data suggest that *B. pertussis*-induced serum does not have inhibitors that interfere with the effects of antibodies.

**Coinfection and antibody-mediated clearance.** Previous experiments have shown that *B. bronchiseptica* induces an innate immune response that is substantially greater than that of *B. pertussis* (4), and we hypothesized that these differences may contribute to antibody-mediated bacterial clearance by increasing the access of antibodies, complement, and FcR-bearing phagocytes to the bacterial micro-colonies in the respiratory tract. If *B. bronchiseptica* induces inflammation that contributes to antibody-mediated bacterial clearance, then inoculation of *B. bronchiseptica* along with *B. pertussis*
would be predicted to allow *B. pertussis*-induced sera to eliminate *B. pertussis*. To test this hypothesis, *B. bronchiseptica* and *B. pertussis* were co-inoculated into wild type mice to which either *B. bronchiseptica-* or *B. pertussis*-induced serum was adoptively transferred. *B. bronchiseptica*-induced serum did not affect *B. pertussis* numbers but rapidly cleared *B. bronchiseptica* from the lower respiratory tract by day 3 postinoculation, again suggesting that *B. pertussis* does not inhibit some antibody function. However, *B. pertussis*-induced serum did not affect the numbers of either *B. pertussis* or *B. bronchiseptica* bacteria in the respiratory tract (Fig. 6), indicating that the inflammatory response elicited by *B. bronchiseptica* did not facilitate serum antibody-mediated clearance of *B. pertussis*. The failure of *B. pertussis*-induced serum to affect *B. bronchiseptica* is discussed below.
DISCUSSION

To better understand immunity to bordetellae, we examined the two common human pathogens *B. pertussis* and *B. parapertussis* alongside *B. bronchiseptica*, which naturally infects mice. Our results show that B cells are required to clear all three *Bordetella* subspecies from the respiratory tracts of mice, in agreement with previous results obtained with *B. pertussis* (8, 9). Adoptive transfer of convalescent-phase serum was sufficient to rapidly clear the lower respiratory tract of the broad-host-range pathogen *B. bronchiseptica* but not the human-specific pathogens *B. pertussis* and *B. parapertussis*. These results obtained with the mouse model are consistent with human clinical trials, in which serum antibody titers could not be correlated with protection against *B. pertussis* and intravenous immunoglobulin therapy had modest effects, thus supporting the validity of the mouse model as accurately reflecting the roles of individual host immune functions (3, 11). Since *B. pertussis* and *B. parapertussis* appear to have emerged independently as human pathogens and each is more closely related to a *B. bronchiseptica*-like progenitor than each is to the other (16), these results raise the possibility that resistance to serum antibodies may relate to their adaptation to humans. Understanding the mechanism(s) involved in avoiding or delaying antibody-mediated clearance may contribute to the development of improved vaccines or treatments for human respiratory infections.

It is well established that *B. bronchiseptica* induces higher serum antibody titers than *B. pertussis* in the mouse model (4), and we hypothesized that qualitative and/or quantitative differences in the antibodies could explain their different in vivo effects. However, *B. parapertussis* and *B. bronchiseptica* induced similar antibody titers but these antibodies completely cleared only the latter without affecting the former. Additionally,
when we compensated for the lower antibody titers of *B. pertussis*-induced serum by increasing the volume delivered, there was still no effect on *B. pertussis*, suggesting that the difference in their antibacterial function is not due to differences in overall antibody titers. It is possible that the antibodies differed qualitatively; however, the distribution of the various antibody isotypes was not significantly different among the three immune sera. Furthermore, antibodies raised against *B. bronchiseptica* also recognized *B. pertussis* and vice versa (5). In addition, each immune serum killed all three organisms in serum killing assays, indicating that they recognized, bound, and activated complement on the surface of each bacterium in vitro (data not shown). Together, these data suggest that differential antibody effects cannot be attributed to quantitative differences in the antibodies but may be due to qualitative differences or in vivo susceptibilities of the organisms.

We have shown that *B. bronchiseptica* induces substantially more inflammation than *B. pertussis* (4) and have recently observed that this inflammation is important in antibody-mediated bacterial clearance (Chapter 3). We therefore hypothesized that the greater inflammation induced by *B. bronchiseptica* might allow anti-*B. pertussis* antibodies to be more effective. Alternatively, *B. pertussis* could inhibit antibody access to, or function within, the respiratory tract. However, co-infection with these two organisms neither increased antibody-mediated clearance of *B. pertussis* nor inhibited clearance of *B. bronchiseptica*. Together, these results suggest that the human-specific bordetellae have a mechanism to resist antibody-mediated clearance that *B. bronchiseptica* does not. Interestingly, *B. pertussis*-induced serum did not affect *B. bronchiseptica* in coinfection experiments, although it did bind and kill *B. bronchiseptica*
in serum killing assays in vitro. Differences in antibody activities have been shown to depend on the isotype, the cognate antigen, and even the epitope on that antigen (13). These parameters may determine whether antibodies are effective or whether they actually interfere with, or block, more effective antibodies (13). We are currently investigating these possibilities.

It is possible that differences in susceptibility to antibodies relate to the different epidemiologies of these organisms. *B. bronchiseptica* can persist for years within the nasal cavity of its host, where serum antibodies have no effect. Individual humans usually eliminate infections by bordetellae, yet *B. pertussis* persists within relatively dense and mobile human populations, including those in which vaccine coverage is very high. This environment would be expected to provide selection for organisms with the ability to infect hosts that were previously exposed via vaccination or prior infection. Therefore, the ability to resist antibody-mediated clearance, possibly acquired by the two human pathogens during their adaptation to a new host, may relate to their epidemiology. It is worth noting that *B. bronchiseptica* occasionally infects humans but does not appear to spread efficiently and is usually associated with immunocompromised individuals (19), perhaps reflecting its susceptibility to antibody-mediated clearance. In the light of these observations, it is possible that acquisition of resistance to antibody-mediated clearance represents an important step in the independent emergence of *B. pertussis* and *B. parapertussis* as human pathogens.

The effects of antibodies on *B. pertussis* in vitro have been well studied, and a number of observations may relate to the ability of this organism to resist antibody-
mediated clearance in vivo. Stefanelli et al. have shown that phagocytosis of *B. pertussis* by a human macrophage-like cell line cannot be enhanced by opsonizing the bacteria with human immune serum in an in vitro assay (14). A similar observation with human neutrophils was made by Weiss et al., who also showed that *B. pertussis* expresses a protein, BrkA, that confers a level of resistance to serum complement in vitro (2, 18). However, our data show that convalescent-phase serum that binds and rapidly kills *B. pertussis* in vitro has no effect when adoptively transferred in vivo, consistent with previous observations with vaccine-induced serum. Therefore, it is difficult to assess the relevance of these, and other, in vitro observations to the effects of antibodies in vivo. Since *B. bronchiseptica* is both closely related to and the apparent progenitor of both human pathogens, understanding the mechanism(s) involved in antibody-mediated clearance of *B. bronchiseptica* will reveal the pathways that are blocked or avoided by *B. pertussis* and *B. parapertussis*. 
FIG. 1. MuMT mice are defective in clearing *B. bronchiseptica*, *B. parapertussis*, and *B. pertussis* from the respiratory tract. Groups of four 4- to 6-week-old C57BL/6 (△) and MuMT (⊙) mice were inoculated with $5 \times 10^5$ CFU of *B. bronchiseptica* (A), *B. parapertussis* (B), or *B. pertussis* (C) delivered in a 50-µl volume of PBS into the nares. The number of bacteria recovered from the nasal cavity, trachea, or lungs at each indicated time postinoculation is expressed as the log$_{10}$ mean ± the standard error.
FIG. 2. Adoptive transfer of serum antibodies clears *B. bronchiseptica* but not *B. parapertussis* or *B. pertussis* from the lower respiratory tract. Groups of four 4- to 6-week-old C57BL/6 mice were inoculated with $5 \times 10^5$ CFU of *B. bronchiseptica* (A), *B. parapertussis* (B), or *B. pertussis* (C) delivered in a 50-µl volume of PBS into the nares. Two hundred microliters of PBS (□), naive serum (○), or convalescent-phase serum (△) was given by intraperitoneal injection prior to inoculation. The number of bacteria recovered from the nasal cavity, trachea, or lungs at each indicated time postinoculation is expressed as the log10 mean ± the standard error.
FIG. 3. Adoptive transfer of serum antibodies clears B. bronchiseptica from the respiratory tracts of MuMT mice. Groups of four 4- to 6-week-old MuMT mice were inoculated with $5 \times 10^5$ CFU of B. bronchiseptica delivered in a 50-µl volume of PBS into the nares. Two hundred microliters of PBS (○), naive serum (△), or convalescent-phase serum (□) was given by intraperitoneal injection prior to inoculation. The number of bacteria recovered from the nasal cavity, trachea, or lungs at each indicated time postinoculation is expressed as the log_{10} mean ± the standard error.
FIG. 4. Characterization of convalescent-phase sera. Anti-*Bordetella* antibody titers were compared in serum samples collected 28 days after intranasal inoculation of mice with a large dose (50 µl of PBS containing $5 \times 10^5$ CFU) of *B. bronchiseptica* (■), *B. parapertussis* (□), or *B. pertussis* (■). Whole cells of the indicated *Bordetella* subspecies were used as the antigen in each enzyme-linked immunosorbent assay. Bound anti-*Bordetella* antibodies were detected by secondary antibodies specific for total and indicated isotypes of immunoglobulin (Ig). Bars represent the means ± the standard errors of the immunoglobulin titers detected. ND, not detectable.
FIG. 5. Increasing the volume of serum does not affect *B. pertussis* numbers in lungs. Groups of four 4- to 6-week-old C57BL/6 mice were inoculated with $5 \times 10^5$ *B. pertussis* bacteria delivered in a 50-µl volume of PBS into the nares. Six hundred microliters of convalescent-phase or naive serum was injected by the intraperitoneal route prior to inoculation. The number of bacteria recovered from the lungs on day 3 postinoculation is expressed as the $\log_{10}$ mean ± the standard error.
FIG. 6. Coinoculation of *B. bronchiseptica* with *B. pertussis* does not affect *B. pertussis* CFU counts in the lungs. Groups of four 4- to 6-week-old C57BL/6 mice were inoculated with $5 \times 10^5$ *B. bronchiseptica* and *B. pertussis* bacteria delivered in 50 µl of PBS. Six hundred microliters of either *B. bronchiseptica*- or *B. pertussis*-induced serum was injected by the intraperitoneal route prior to inoculation. The number of *B. bronchiseptica* (■) or *B. pertussis* (□) bacteria recovered from the lungs on day 3 postinoculation is expressed as the log$_{10}$ mean ± the standard error.
REFERENCES


CHAPTER 3

IgA IS ESSENTIAL FOR IMMUNITY TO *BORDETELLA BRONCHISEPTICA*, BUT NOT *B. PERTUSSIS* AND *B. PARAPERTUSSIS*
ABSTRACT

The belief that secretory IgA is critical in preventing pathogen entry and colonization of a host has prompted the development of vaccines that induce a strong IgA response at mucosal surfaces. Here we evaluated the role of IgA in respiratory infections caused by *Bordetella*. IgA<sup>−/−</sup> mice were able to clear the animal pathogen *B. bronchiseptica* from the lungs. The reduction in bacterial numbers in the trachea and nasal cavity of IgA<sup>−/−</sup> mice was, however, significantly delayed compared to that of wild type mice. Similarly, convalescent IgA<sup>−/−</sup> mice failed to prevent *B. bronchiseptica* colonization in the nasal cavity and trachea upon a secondary challenge. In addition, convalescent-phase serum from IgA<sup>−/−</sup> mice was defective in clearing the bacterium from the trachea. Together these data suggest that IgA is important for host immunity in the upper respiratory tract but not in the lungs. Interestingly, IgA<sup>−/−</sup> mice, similar to wild type mice, cleared the human pathogens *B. pertussis* and *B. parapertussis* from the respiratory tract and IgA was not required for infection-induced immunity to secondary challenge with these human pathogens. These data suggest that non-IgA immune responses are sufficient to control human adapted bordetellae.
INTRODUCTION

Clinical findings indicate that people with IgA deficiencies have higher chances of contracting sinopulmonary and gastrointestinal tract infections suggesting that IgA plays a significant role in controlling mucosal pathogens (3). In animal studies, IgA has been shown to be necessary for immunity to gram-positive bacteria such as *Streptococcus pneumoniae* (25). Arulanandam et al have demonstrated the need for IgA in protective anti-influenza immunity (1). Recently, Renegar et al have demonstrated that transfer of IgA, but not IgG, effectively reduced nasal carriage of influenza virus in a mouse model (22). These findings are consistent with clinical reports indicating individuals with primary IgA deficiency are more susceptible to recurrent streptococcal and influenza infections (4). However, the contribution of IgA in immunity to gram-negative bacteria is not fully understood.

*Bordetella bronchiseptica* is a gram-negative mucosal pathogen that naturally colonizes the upper respiratory tract of mice and as such is used as a model to study natural host-pathogen interactions (9). *B. pertussis* and *B. parapertussis* are closely related to *B. bronchiseptica* but primarily infect humans (26), although the mouse model has been extensively used to study immune responses to these human adapted bordetellae (19). Previously we have shown that, although B cells are required for clearance of *Bordetella* in mice, adoptively transferred serum antibodies had no effect on bacteria in the nasal cavities (15). Since serum contained very low titers of IgA, we hypothesized that IgA may be important in controlling bacteria in the upper respiratory tract. Here we analyzed the role of IgA in immunity to various bordetellae and demonstrate that IgA is essential for controlling *B. bronchiseptica* in the upper respiratory tract of mice. We also
demonstrate that IgA is essential for preventing secondary infection with *B. bronchiseptica*. Interestingly, IgA was not required for protective immunity to human adapted bordetellae.
MATERIALS AND METHOD

Bacteria and mice

*B. bronchiseptica* RB50\(^{\text{Str}}\) is a streptomycin resistant strain isolated from rabbit (9). BP 536\(^{\text{Str}}\) is a streptomycin resistant derivative of Tohama I, a clinical isolate of *B. pertussis* and BPP12822 is a *B. parapertussis* clinical isolate (9, 10). RB50\(^{\text{Gent}}\), BP536\(^{\text{Gent}}\) and BP12822\(^{\text{Gent}}\) were created by inserting GentR cassette into Bvg\(^{-}\) gene *fla* of RB50, BP536, and BPP12822. Bacteria were maintained on Bordet-Gengou (BG) agar (Difco) supplemented with 7.5% defribinated sheep’s blood (Remel or Hema Resources) and 20 \(\mu\)g/ml streptomycin or gentamicin (20\(\mu\)g/ml). Bacteria were grown in Stainer-Scholte (SS) broth with supplements and 20 \(\mu\)g/ml streptomycin to mid-log phase (optical densities of approximately 0.3 at 600 nm) at 37\(^\circ\)C on a roller drum for experiments.

C57BL/6 mice were obtained from Jackson Laboratories and IgA\(^{-/-}\) mice were kind gift from Innocent Mbwaukee, Baylor college of Medicine, Houston, TX and are described elsewhere (8).

Inoculation and adoptive transfer protocols

Mice were lightly sedated with isoflurane (IsoFlo-Abbott Laboratories), and 5 X 10\(^{5}\) CFU of bacteria in 50 \(\mu\)l of PBS were inoculated onto the tip of the external nares. For the time course experiment, groups of four animals were sacrificed on days 0, 7, 14, 28, 49, 70, and 105 post-inoculation. Colonization levels were determined by homogenizing the indicated organs in 1 X PBS and plating aliquots for colony counts. The nasal cavity and trachea were homogenized in 500 \(\mu\)l of PBS and lungs in 1 ml PBS. The homogenates and necessary dilutions were plated in 50 \(\mu\)l volumes onto BG agar with streptomycin and/or gentamicin. Colonies were counted after 2 (RB50), 3 (BP536), and 4 (BPP 12822) days incubation at 37\(^\circ\)C. For adoptive transfer experiments,
intraperitoneal injection of 200 µl of convalescent-phase serum obtained on day 28 post-inoculation from mice which were inoculated with RB50^StrR, BP536^StrR or BPP12822, into naïve mice was immediately followed by inoculation as described above. Animals were sacrificed on the indicated day post transfer and inoculation and colonization determined as described. Animals were handled in accordance with institutional guidelines. Statistical significance of data points was determined using a student's unpaired t-test.

**Challenge protocol**

For experiments with *B. bronchiseptica*, mice were inoculated with 5 x 10^5 CFU of bacteria as described above. These mice were treated with gentamicin via drinking water (10mg/ml) for 7 days starting on day 21 post-inoculation. On day 28 post-inoculation, mice were challenged with 5 x 10^5 CFU of RB50^GentR. For experiments with *B. pertussis* and *B. parapertussis*, mice were inoculated with 5 X 10^5 CFU of either BP 536^StrR or BPP12822 as described above. Mice were treated with gentamicin for 3 days starting on day 25 post-inoculation. These mice were subsequently challenged with 5 x 10^5 CFU of either BP 536 or BPP12822^GentR on day 28 post-inoculation.

**Antibodies**

Titers of anti-*Bordetella* antibody in convalescent-phase sera were determined by enzyme-linked immunosorbent assay with polyvalent anti-mouse secondary antibodies as described previously (13). Specific classes and isotypes of antibodies were determined by using appropriate secondary antibodies (Southern Biotechnology Associates, Birmingham, AL and Pharmingen, San Diego, CA).
RESULTS

IgA is required for the reduction in numbers of *B. bronchiseptica* in the upper respiratory tract

We have previously shown that B cell deficient mice fail to clear *B. bronchiseptica* from the respiratory tract suggesting antibodies are critical for bacterial clearance (15). However, adoptive transfer of serum antibodies cleared *B. bronchiseptica* from the lungs and trachea but not nasal cavities (15) suggesting local antibodies such as IgA may play an important role in the reduction of bacterial numbers in the upper respiratory tract. To address this hypothesis, wild type and IgA−/− mice were inoculated with 5 × 10^5 CFU of *B. bronchiseptica* in 50 μl of PBS by intranasal route. Mice were sacrificed and the respiratory organs were collected for enumerating the bacteria on days 3, 7, 14, 28, 49, 70, and 105 post-inoculation. IgA−/− mice cleared *B. bronchiseptica* in the lungs in a pattern indistinguishable from that of wild type mice (Fig. 1). IgA−/− mice began to reduce the number of bacteria in the tracheas and nasal cavities only after day 28 while wild type mice began to reduce the number as early as day 7 post-inoculation. Approximately 10 – 100 fold higher numbers of bacteria were detected in the tracheas of IgA−/− mice than that of wild type mice on days 14, 28, and 49. However, IgA−/− mice completely cleared bacteria in the trachea by day 105 post-inoculation. A similar but more dramatic defect in the bacterial clearance was observed in the nasal cavities of IgA−/− mice. While wild type mice reduced the bacterial numbers in the nasal cavities to ~1000s on day 105, IgA−/− mice harbored ~100,000 CFU. These results indicate that IgA is critical in the reduction of *B. bronchiseptica* numbers in the trachea and nasal cavity but not in the lungs.
IgA is critical for sterilizing immunity to *B. bronchiseptica*

In order to control the spread of bacteria, it is critical for a *B. bronchiseptica* vaccine to provide sterilizing immunity in the upper respiratory tract, the natural place of colonization for this bacterium. We have previously observed that immunity conferred by prior infection is superior to parenteral vaccination in preventing colonization by *B. bronchiseptica* (Lakshmi Gopinathan and Harvill E.T, Unpublished results). Interestingly, infection, but not parenteral vaccines, induces a significant IgA response suggesting that mucosal IgA may be important in immunity to *B. bronchiseptica* in the nasal cavity (Lakshmi Gopinathan and Harvill E.T., Unpublished results). In order to address this possibility, wild type and IgA⁻/⁻ mice were inoculated with *B. bronchiseptica* StrR as described in materials and methods section. Beginning on day 21, post-inoculation, mice were treated with Gentamicin for 7 days. This treatment completely eliminated bacteria in the lungs and trachea whereas only 10-100 bacteria remained in the nasal cavities on day 28 post-inoculation. These mice were challenged with 5 X 10⁵ CFU of *B. bronchiseptica* GentR on day 30 post-inoculation as described previously. On day 3 post-challenge, the number of *B. bronchiseptica* (both StrR and GentR) recovered from various respiratory organs were measured. No *B. bronchiseptica* could be recovered from the lungs of either wild type or IgA⁻/⁻ mice (Fig. 2). While the tracheas of IgA⁻/⁻ mice harbored 1000s of *B. bronchiseptica* GentR, no bacteria could be recovered from the tracheas of wild type mice. Similarly, the nasal cavities of IgA⁻/⁻ mice harbored 1000s of *B. bronchiseptica* StrR and 100,000s of *B. bronchiseptica* GentR, whereas that of wild type mice harbored only 100s of *B. bronchiseptica* StrR and no *B. bronchiseptica* GentR. A similar result was also observed on day 7 post-challenge (data not
shown), indicating that IgA<sup>−/−</sup> mice fail to prevent *B. bronchiseptica* colonization in the tracheas and nasal cavities upon challenge.

**IgA<sup>−/−</sup> mice show normal serum antibody response**

IgA<sup>−/−</sup> mice have an altered antibody isotype profile such as increased IgG3 in response to influenza viral infection (8, 29). One of the possible explanations for the inability of IgA<sup>−/−</sup> mice to clear primary or secondary *B. bronchiseptica* infection in the tracheas and nasal cavities could be that these mice may have an altered serum antibody response. Therefore, we compared the titers of *Borrelia*-specific antibodies and isotypes of wild type and IgA<sup>−/−</sup> mice inoculated with *B. bronchiseptica* and sera collected on day 28 post-inoculation. The overall titers of serum antibodies in IgA<sup>−/−</sup> mice were not significantly different from that of wild type mice (Fig. 3). Similarly, titers of various isotypes of anti-*Borrelia* antibodies were also indistinguishable between wild type and IgA<sup>−/−</sup> mice. These results indicate that IgA<sup>−/−</sup> mice developed a normal serum antibody response and suggest that mucosal IgA is necessary for reducing the numbers of *B. bronchiseptica* in the trachea and nasal cavities.

**Serum antibody-mediated clearance of *B. bronchiseptica* in the trachea requires IgA**

Previously we have shown that adoptively transferred serum antibodies rapidly clear *B. bronchiseptica* from the lung and trachea of mice (15). Here we sought to determine the contribution of serum IgA in bacterial clearance. Convalescent-phase serum from wild type and IgA<sup>−/−</sup> mice inoculated with *B. bronchiseptica* was collected on day 28 post-inoculation. Groups of wild type mice were inoculated with *B. bronchiseptica* as described previously and adoptively transferred 200µl of naïve serum or convalescent-phase serum from wild type or IgA<sup>−/−</sup> mice. Naïve serum treated mice
harbored approximately $10^6$ CFU and $10^4$ CFU of *B. bronchiseptica* in the lungs and trachea, respectively, on day 3 post-inoculation. Wild type serum cleared *B. bronchiseptica* in the lungs and trachea on day 3 post-inoculation (Fig. 4). IgA deficient serum, however, cleared *B. bronchiseptica* in the lungs but not trachea indicating that serum IgA is required for bacterial clearance in the trachea.

**IgA is not required for control of human adapted *Bordetella***

Both *B. pertussis* and *B. parapertussis* require B cells for their clearance from the respiratory tract of mice (15, 17, and 18). However, adoptively transferred serum antibodies have no effect on bacterial numbers in the upper respiratory tract of mice infected with these two sub species (15). Furthermore, adoptive transfer of serum antibodies has no effect on bacterial numbers in the respiratory tract for 7 days post-inoculation (15). Therefore, we determined the contribution of IgA in the clearance of human adapted *Bordetella*. Wild type and IgA$^{-/-}$ mice were inoculated with $5 \times 10^5$ CFU of either *B. pertussis* or *B. parapertussis* by intranasal route and the number of bacteria in the lungs, trachea and nasal cavities was determined on subsequent days. In contrast to *B. bronchiseptica*, the number of *B. pertussis* recovered from the lungs, trachea and nasal cavities of IgA$^{-/-}$ mice were similar to that recovered from wild type mice on days 7, 14, 28, and 49 post-inoculation (Fig. 5A). Wild type and IgA$^{-/-}$ mice cleared bacteria from all three respiratory organs by day 70 post-inoculation. Similarly, IgA$^{-/-}$ mice were near normal in controlling and clearing *B. parapertussis* in the lungs and trachea and in reducing the number of bacteria in the nasal cavities (Fig. 5B). Evaluation of serum antibody titers and isotypes did not reveal a significant difference between wild type and IgA$^{-/-}$ mice infected with either *B. pertussis* or *B. parapertussis* (data not shown). These
results indicate that IgA is not essential for the natural clearance of *B. pertussis* and *B. parapertussis* in mice.

**IgA is not required for anamnestic immune response to human adapted Bordetella**

In contrast to vaccines, infection is thought to provide long lasting immunity to *B. pertussis* (27). Interestingly, previously infected, but not vaccinated, humans have anti-*B. pertussis* IgA in their sera prompting many investigators to believe that mucosal vaccines that can induce IgA may provide better protection than the current parenteral vaccines (12). Furthermore, it is thought that reciprocal protection against *B. pertussis* and *B. parapertussis* is also mediated via IgA (28). We sought to determine the importance of IgA in immunity to *B. pertussis* and *B. parapertussis*. Wild type and IgA−/− mice were inoculated with either *B. pertussis* or *B. parapertussis* as described previously. Beginning on day 25 post-inoculation, mice were treated with gentamicin for 3 days after which no *Bordetella* could be recovered from these mice. On day 30 post-inoculation, mice were challenged with 5 X 10^5 CFU of either *B. pertussis*<sub>GentR</sub> or *B. parapertussis*<sub>GentR</sub> as described previously. Both wild type and IgA−/− mice cleared challenge bacteria on day 7 post-inoculation (Fig. 6A and 6B) indicating that IgA is not required for immunity to *B. pertussis* and *B. parapertussis*. 
DISCUSSION

Bacteria such as *Streptococcus, Neisseria*, and *Bordetella* continue to circulate in spite of the widespread use of vaccines (5, 6, and 7). This may, in part, be attributed to current vaccine strategies which are aimed at inducing serum antibodies to specific virulence factors and therefore protect against severe disease but do not prevent infection. Similarly, we and others have observed that serum antibodies do not affect mucosal pathogens in the upper respiratory tract (15). It is therefore important to elucidate the local immune responses, which can eliminate bacteria from their natural site of infection in order to aid in the design of novel vaccines that not only protect from disease but also prevent infection.

Bacterial/viral infections that induce mucosal immunity such as a high IgA response have been shown to provide long lasting protection from re-infection (14). Local adaptive immune responses, such as IgA, provide the first line of defense by neutralizing and agglutinating pathogens at the mucosal surface. Recently, IgA has also been implicated in modulating local inflammation and thus preventing the spread of pathogens (20). IgA has been shown to be critical in immunity to mucosal pathogens such as *Streptococcus pneumoniae* and the influenza virus (1, 22, and 25). Based on the assumption that IgA may also be critical for protective immunity to other mucosal pathogens, recent immunization strategies emphasize the induction of a strong local IgA response using mucosal vaccines and a variety of adjuvants such as cholera toxin (23).

Little is known about the role of IgA in *Bordetella* infections. Using *B. bronchiseptica*-mouse model, we demonstrate that IgA is required for sterilizing immunity in the nasal cavity and trachea but not in the lungs. Interestingly, *B.
*B. bronchiseptica* naturally colonizes and chronically persists in the nasal cavity and trachea of a variety of mammals (9). Therefore, parenteral vaccines, which induce serum antibodies, may have limited effect in preventing *B. bronchiseptica* infection. In fact, many clinical studies have shown that *B. bronchiseptica* can be isolated from vaccinated pig farms (24). It is possible that the use of intranasal vaccines that induce a robust mucosal IgA response in the upper respiratory tract could provide sterilizing herd immunity.

Individuals infected with *B. pertussis* have elevated level of IgA in their sera prompting some investigators to suggest an association between serum IgA and a long lasting immunity observed post-infection (12). Watanabe et al have showed that pre-incubation of *B. pertussis* with BAL fluid from *B. parapertussis* infected mice, or vice versa, before inoculation augments their clearance, leading the authors to conclude that IgA is involved in this reciprocal immunity (28). Similarly, Helwigg et al have shown that pre-incubation of *B. pertussis* with human IgA before inoculation hastens bacterial clearance in transgenic mice expressing human FcRα (11). However, in this report we demonstrate that IgA is not necessary for natural immunity to *B. pertussis* or *B. parapertussis*. These data differ from previously published results which suggest that IgA is important in mucosal immunity to *B. pertussis* and *B. parapertussis* (11, 28). It is worth noting that those previous results were obtained by adsorbing bacteria with antibodies before inoculation which may interfere with the attachment of bacteria in the respiratory tract. Consistent with our findings, IgA deficiencies have not been associated with increased incidence of whooping cough (4). Additionally, infection-induced immunity induces a robust Th1 type response which has been shown to contribute to long
lasting immunity against *B. pertussis* (2). Furthermore, clinical trials and our recent experimental evidence suggest that anti-pertussis toxin serum antibodies are critical for sterilizing immunity (Chapter 5 and 16).

Proposed strategies to prevent *B. pertussis* circulation include the use of mucosal pertussis vaccines that induce a strong IgA response. Additional advantages of such vaccines are thought to include cross protection against *B. parapertussis*, to which no vaccines are available. Although we can not rule out a role for IgA in immunity to *B. pertussis or B. parapertussis*, our results demonstrate that IgA is not necessary for sterilizing immunity to *B. pertussis or B. parapertussis*. Consistent with earlier reports, our data indicate a strong serum antibody response, along with cell-mediated immunity, appears to be sufficient for immunity to bordetellae in the lower respiratory tract (7, 16, and 17), the natural sites of colonization of human adapted bordetellae.
Fig. 1 IgA is required to reduce the numbers of *B. bronchiseptica* in the upper respiratory tract. Groups of 4 mice each of C57BL/6 and IgA\(^{-/-}\) mice were inoculated with 50\(\mu\)l of PBS containing 5 x 10^5 CFU of *B. bronchiseptica*. Lungs, trachea and nasal cavities were harvested on days 0, 7, 14, 28, 49, 70, and 105 post-inoculation and the numbers of bacteria were measured in each organ and expressed as mean ± SE.
Fig. 2 IgA is required to prevent colonization of secondary infection by *B. bronchiseptica*. Groups of 4 each of C57BL/6 and IgA/−/− mice were inoculated with 5 × 10^5 CFU *B. bronchiseptica*StrR in 50µl of PBS. These mice were treated with Gentamicin for 7 days beginning on day 21 post-inoculation. On day 30, mice were challenged with 5 × 10^5 CFU of *B. bronchiseptica*GentR. Mice were sacrificed on day 3 post-challenge and various respiratory organs were harvested. The number of *B. bronchiseptica*GentR and *B. bronchiseptica*StrR were measured in the lungs, trachea and nasal cavity. The numbers of *B. bronchiseptica*GentR in each organ are expressed as mean ± SE.
Fig. 3 Antibody titers of convalescent-phase serum from wild type and IgA−/− mice inoculated with *B. bronchiseptica*. Groups of C57BL/6 and IgA−/− mice were inoculated with 5 X 10⁵ CFU of *B. bronchiseptica* in 50µl of PBS. On day 28 post-inoculation, sera were collected from these mice and titers of anti-*Bordetella* antibodies were determined by ELISA and expressed as mean ± SE.

![Bar graph showing antibody titers](image-url)
Fig. 4 IgA deficient convalescent-phase serum is effective in reducing *B. bronchiseptica* in the lungs but not in the trachea or nasal cavity. Groups of C57BL/6 mice were inoculated with $5 \times 10^5$ CFU of *B. bronchiseptica* in 50µl of PBS. Subsequently, they were adoptively transferred 200µl of naïve, wild type or IgA$^{-/-}$ convalescent phase serum by intraperitoneal route. On day 3 post-inoculation, various respiratory organs were harvested and bacterial numbers were measured in the lungs, trachea and nasal cavity.
Fig. 5 IgA is not required for natural clearance of *B. pertussis* or *B. parapertussis*.

Groups of 4 mice each of C57BL/6 and IgA−/− mice were inoculated with 50μl of PBS containing 5 X10^5 CFU of *B. pertussis* or *B. parapertussis*. Lungs, trachea and nasal cavities were harvested on days 0, 3, 7, 14, 28, 49, 70, and 105 post-inoculation and the numbers of bacteria were measured in each organ and expressed as mean ± SE.
Fig. 6 IgA is not required for anamnestic response to *B. pertussis* or *B. parapertussis*. Groups of 4 each of C57BL/6 and IgA−/− mice were inoculated with 5X10^5 CFU *B. pertussis*<sup>StrR</sup> or *B. parapertussis*<sup>StrR</sup> in 50μl of PBS. These mice were treated with Gentamicin for 3 days beginning on day 25 post-inoculation. On day 30, mice were challenged with 5 X10^5 CFU of *B. pertussis*<sup>GentR</sup> or *B. parapertussis*<sup>GentR</sup>. Mice were sacrificed on day 3 post-challenge and various respiratory organs were harvested. The number of *B. pertussis*<sup>GentR</sup> and *B. Parapertussis*<sup>GentR</sup> were measured in the lungs, trachea and nasal cavity. The numbers of bacteria in each organ are expressed as mean ± SE.
REFERENCES


CHAPTER 4

ANTIBODY-MEDIATED CLEARANCE OF *BORDETELLA*

FROM THE LUNGS REQUIRES TLR4 AS WELL AS

*FCγRS, C3 AND CR3.*
ABSTRACT

Parenteral vaccination generally induces high serum antibody titers, however these antibodies have variable effects on the elimination of pathogens within the respiratory tract. In this study, we investigated the specific mechanism of serum antibody-mediated clearance of mouse respiratory pathogen *B. bronchiseptica*. Our data indicate that antibody-mediated clearance requires complement not for direct bacterial lysis, but for CR3-mediated phagocytosis. Fcγ receptors are also required in the lungs but a single FcγR was sufficient. FcγR and CR3 bearing neutrophils were found to be critical for bacterial clearance. Interestingly, the innate immune receptor TLR4 was also found to be required for antibody-mediated protection, primarily for the recruitment of neutrophils. These results suggest a model in which TLR4-mediated inflammatory responses aid in the recruitment of cells such as PMNs, which phagocytose the opsonised bacteria via FcγRs and CR3. Complement, but not FcγRs, is required for antibody-mediated bacterial clearance in the trachea, suggesting the presence of differential and organ-specific immune requirements. Understanding the mechanism by which serum antibodies can clear bacteria may lead to the design of vaccines that optimize protection and guide studies of pathogen strategies to avoid clearance.
INTRODUCTION:

Serum antibodies play a critical role during natural infection in eliminating both intracellular and extracellular pathogens (40). Majority of vaccines are also designed to generate high titers of serum antibodies. However, little is known about the mechanism by which these antibodies clear pathogens, more so in the case of respiratory pathogens. With the recent advocating of use of passive transfer of antibodies in the case of bioterrorist attack with variety of bacterial and viral agents (3), it is even more important to understand the mechanism by which antibodies clear a pathogen. This could aid in the design of appropriate immunizing agents for prophylaxis and therapeutics.

Classically, antibodies are known to act on a pathogen and eliminate it by three mechanisms: neutralization, opsonization and phagocytosis, and complement activation (40). Even though there are instances of elimination of pathogens by combination of these effects (40), it is not clear how various immune arms interact and coordinate in the removal of bacteria from the respiratory tract. Furthermore, majority of the studies focused on effect of antibodies use in vitro systems and mostly monoclonal antibodies (15). However, the actual in vivo mechanism is likely to be more complex and different. It is difficult to model variety of immune functions that can interact in the elimination of a pathogen in in vitro systems. Therefore, caution should be exercised in interpreting in vitro data in natural infection. For instance, in vitro studies suggest that monoclonal antibodies of the IgG3 isotype raised against capsular antigens of Streptococcus pneumoniae act by activating complement and lysing cells via membrane attack complex formation (31). However, in vivo studies with this pathogen indicate limited importance of IgG3 in bacterial clearance raising doubts about the relevance of in vitro data to in
vivo infection (24). During natural infection serum contains multiple antibody isotypes that may compete with or augment IgG3 mediated bacterial clearance (24). Availability of good natural host-pathogen model can address this problem and provide useful insights into the in vivo mechanisms of antibody-mediated bacterial clearance.

*Bordetella bronchiseptica* is a gram-negative bacterium that causes respiratory diseases in many non-human mammals (28). The bacterium naturally infects mice and as such this model can be used to study natural host-pathogen interactions (16). Previously, we have shown that adoptively transferred serum antibodies are sufficient to clear these bacteria from the lower respiratory tracts of mice (20). Thus adoptive transfer approach along with the use of mice lacking specific immune functions can be applied to examine the mechanism by which antibodies eliminate these bacteria from the respiratory tract (40).

Respiratory system comprises of complex tissues which needs to be relatively sterile in spite of exposure to heavy load of pathogens. This is partly achieved by innate immune systems including physical barriers and mechanical forces (1). Recently discovered pattern recognition receptors play a major role in recognizing and mounting an immediate immune response against specific pathogens (29). Toll like receptor-4, one such receptor, is well studied and is known to recognize LPS of gram-negative bacteria and induce a potent pro-inflammatory response (4, 17, and 39). In fact TLR4 is critical for controlling many respiratory pathogens such as *Pseudomonas aeruginosa*, *Haemophilus influenzae* and more recently *B. bronchiseptica* (10, 23, and 39). Although, many studies have described the involvement of TLR4 in the generation of adaptive immunity (25), their role in effectiveness of adaptive immunity is less understood. Here
we describe the potential role for TLR4 in the effect of antibodies than in the generation of antibody response.

Our evaluation of antibody functions required for bacterial clearance in the respiratory tract indicates that complement is required predominantly for opsonization and that CR3 and FcγRs are required for phagocytosis. Furthermore, neutrophils that express these receptors are recruited via a TLR4 dependant response and are critical for antibody-mediated clearance of bacteria. Together, these data suggest a model for antibody-mediated bacterial clearance in which TLR4 dependant inflammatory response recruits neutrophils to the lungs where they phagocytose antibody and iC3b opsonized bacteria via FcγRs and CR3. These data indicate that effector functions of antibodies are more complex in vivo than proposed by in vitro studies and suggest the close involvement of innate and adaptive immune functions in clearing a pathogen from the body.
MATERIALS & METHODS

Bacteria

*B. bronchiseptica* RB50 (wild type) was obtained after a single passage from an original rabbit isolate (16). Bacteria were maintained on Bordet-Gengou (BG) agar (Difco) supplemented with 7.5% defibrinated sheep’s blood (Remel or Hema Resources) and 20 µg/ml streptomycin. Bacteria were grown in Stainer-Scholte (SS) broth with supplements and 20 µg/ml streptomycin to mid-log phase (optical densities of approximately 0.3 at 600 nm) at 37ºC on a roller drum for experiments.

Mice

C57BL/6 mice were obtained from Jackson Laboratories and C3^−/− mice, back-crossed extensively onto a C57BL/6 background, have been described elsewhere and were kind gifts of Dr. Rick Wetsel (5). C5^−/− mice (B10.D2-H2^dH2-T18^cHc^0/nSnJ) and their parent strain (B10.D2-H2^dH2-T18^cHc^1/nSnJ) were obtained from Jackson Laboratories. FcγR^−/− and FcγRI and FcγRIII (FcγRI^−/− & III^−/−), FcγRII^−/− mice (C57BL/6 background) were obtained from Taconic and are described elsewhere (30, 35, and 36). CD11b^−/− (CR3^−/−) mice were purchased from Jackson laboratories and are described elsewhere (21). TLR4^+^ mice (C3H/HeN) mice were obtained from Charles River laboratories and TLR4^d^ mice (C3H/HeJ) were obtained from Jackson laboratories. The results in C3H/HeN and C3H/HeJ mice were verified in TLR4^−/− mice that were obtained from Jackson laboratories.

Inoculation, vaccination, and adoptive transfer protocols

Mice were lightly sedated with isoflurane (IsoFlo-Abbott Laboratories), and 5 X 10^5 CFU (except where otherwise stated) of bacteria in 50 µl onto the tip of the external nares. For the time course experiment groups of four animals were sacrificed on days 0,
Colonization levels were determined by homogenizing the indicated organ in 1 X PBS and plating aliquots for colony counts. The nasal cavity and trachea were homogenized in 500 µl of PBS and lungs in 1 ml PBS. The homogenates and necessary dilutions were plated in 50 µl volumes onto BG agar with streptomycin. Colonies were counted after 2 days incubation at 37ºC. For re-infection, animals were inoculated with 5 X 10² CFU in 5-µl of PBS by intranasal route. 28 days later these mice were challenged with 5 X 10⁵ CFU of *B. bronchiseptica* in 50-µl of PBS by intranasal route. On day 3 post-challenge mice were sacrificed and colonization levels determined as described above. For vaccination, animals were immunized intraperitoneally (i.p.) twice at two week intervals with 10⁸ CFU of heat-killed *B. bronchiseptica*. Heat-killed bacteria were prepared by incubating bacteria grown to mid-log phase at 80ºC for 30 minutes. Two weeks after administration of the second vaccination, animals were challenged i.n. as described above and sacrificed on day 3 post-challenge for determination of colonization levels as described. Intraperitoneal injection of 200 µl of convalescent-phase serum obtained on day 28 post-inoculation from mice which were inoculated with RB50, into naïve mice was immediately followed by inoculation as described above. Animals were sacrificed on the indicated day post transfer and inoculation and colonization determined as described. Animals were handled in accordance with institutional guidelines. Statistical significance of data points was determined using a student's unpaired t-test.

**Enumeration of leukocytes in the lungs**

Total leukocytes were isolated from the lungs after collagenase and DNAse I digestion as described previously (34). Briefly, lungs were perfused with PBS and finely
sheared by pair of scissors. This lung homogenate was subjected to collagenase type I and Dnase I treatment for about 3 hrs. The enzymatically treated homogenate was laid over Histopaque 1119 (Sigma Aldrich, MO) and centrifuged for 30 min at 3000 rpm. Leukocyte portion was collected and the total number of cells was determined by hemocytometer. Individual cell types were determined by staining the isolated cells with modified Giemsa stain by a certified clinical laboratory technician.
RESULTS

TLR4 is required for antibody-mediated clearance of bacteria from the respiratory tract.

Pattern recognition receptors are known to play a significant role in innate immunity to pathogens (26). We have previously shown that one such receptor, TLR4 is critical for controlling the respiratory pathogen *B. bronchiseptica* (22). TLR4 deficient (TLR4\textsuperscript{d}) mice inoculated with a standard dose of *B. bronchiseptica* succumb to bordetellosis as early as day 3 post-inoculation while wild type (TLR4\textsuperscript{s}) mice survive and eventually clear bacteria from the lower respiratory tract (23). TLR4\textsuperscript{s} mice harbored 1,000,000 CFU in the lungs and 10,000 CFU in the trachea, whereas, TLR4\textsuperscript{d} mice harbored 100 to 1000 fold more CFU in the trachea and lungs on day 3 post-inoculation (Fig. 1A). TLR4\textsuperscript{d} mice succumb to as few as 100 CFU of bacteria if delivered into the lungs indicating TLR4 is essential for innate immunity to the bacteria.

Since TLRs are believed to be involved in generation of adaptive immunity (25), we sought to investigate the importance of TLR4 in adaptive immunity to respiratory pathogens. TLR4\textsuperscript{d} mice were able to clear bacteria from the lower respiratory tract by day 28 post-inoculation when inoculated with extremely low doses (100 CFU in 5-µl PBS) delivering bacteria only to the nasal cavity (unpublished) suggesting TLR4 is not essential for bacterial clearance in convalescent mice. Convalescent TLR4\textsuperscript{s} and TLR4\textsuperscript{d} mice were challenged with our standard dose of bacteria on day 28 post-inoculation. Convalescent TLR4\textsuperscript{s} mice cleared bacteria from the trachea and lungs by day 3 post-challenge (Fig. 1A) suggesting infection-induced adaptive immunity is effective in preventing bacterial colonization. Similarly, convalescent TLR4\textsuperscript{d} mice cleared bacteria
from the trachea and lungs by day 3 post-challenge suggesting TLR4 is not essential for
generation of effective infection-induced immunity.

In order to investigate the requirement for TLR4 for vaccine-induced immunity,
TLR4s and TLR4d mice were intraperitoneally vaccinated with 10^8 heat-killed B.
bronchiseptica and boosted with a second dose 14 days later. On day 28 post-vaccination,
mice were challenged with B. bronchiseptica by our standard regimen and the trachea
and lungs were harvested 3 days later to assess bacterial numbers. TLR4s mice cleared
bacteria from these sites, whereas TLR4d mice harbored approximately 10,000 CFU in
the trachea and 10,000,000 CFU in the lungs (Fig. 1A). Together, these data indicate that
TLR4 is critical for vaccine-induced immunity but not for infection-induced immunity.

Parenteral vaccines generate a high serum antibody response and we have shown
that antibodies are sufficient to rapidly clear B. bronchiseptica from the trachea and lungs
of TLR4s mice (20). We hypothesized that failure of vaccinated TLR4d mice to control
bacteria upon challenge may be due to a defective antibody response. Serum was
collected from vaccinated TLR4s and TLR4d mice on day 28 post-vaccination and anti-
Bordetella antibody titers were determined. No significant difference in antibody titers
was observed between the sera from vaccinated TLR4s and TLR4d mice (Fig. 1B). To
determine if the serum from vaccinated TLR4d mice is less effective than TLR4s wild
type serum in clearing bacteria, wild type mice were inoculated with bacteria and
adoptively transferred 200-µl of convalescent-phase serum or vaccine-induced serum
from either TLR4s or TLR4d mice. Mice were sacrificed on day 3 post-inoculation and
the number of bacteria recovered from the trachea and lungs was enumerated. TLR4d
vaccine serum, similar to TLR4s or TLR4d convalescent-phase serum, cleared the bacteria
from both the trachea and lungs of wild type mice indicating the failure of vaccinated TLR4\textsuperscript{d} mice to clear \textit{B. bronchiseptica} is not due to a defect in antibody generation (Fig. 1C).

The failure of vaccination to induce protection in TLR4\textsuperscript{d} mice could be explained by a defect in antibody functions in these mice. TLR4\textsuperscript{s} and TLR4\textsuperscript{d} mice were inoculated with \textit{B. bronchiseptica} by our standard regimen and adoptively transferred 200-µl of PBS, naïve or convalescent-phase serum at the time of inoculation. TLR4\textsuperscript{s} mice treated with PBS or naive serum harbored approximately $10^7$ CFU of \textit{B. bronchiseptica} in the lungs and $10^5$ CFU in the trachea on day 3 post-inoculation, indicating that naive serum has no significant effect on bacterial numbers (Fig. 2A). TLR4\textsuperscript{d} mice treated with PBS or naïve serum harbored about 100 to 1000 fold more bacteria than that of TLR4\textsuperscript{s} mice in the lungs, and trachea on day 3 post-inoculation (Fig. 2A). TLR4\textsuperscript{s} mice treated with convalescent-phase serum significantly reduced the number of bacteria from the trachea and lungs on day 3 post-inoculation (2A). Surprisingly, TLR4\textsuperscript{d} mice treated with convalescent-phase serum failed to reduce the bacterial numbers significantly (Fig. 2A), suggesting TLR4 is required for antibody-mediated clearance of bacteria from the respiratory tract.

Since the bacterial burdens in TLR4\textsuperscript{d} mice are approximately 1000 folds higher than in TLR4\textsuperscript{s} mice on day 3 post-inoculation, it is possible that the total amount of antibodies transferred was not sufficient to eliminate the very high numbers of bacteria in TLR4\textsuperscript{d} mice. To address this possibility we inoculated TLR4\textsuperscript{s} and TLR4\textsuperscript{d} mice with approximately 500 CFU of \textit{B. bronchiseptica} in 50µl of PBS. With this inoculation regimen, $10^5$-$10^6$ CFU of \textit{B. bronchiseptica} were recovered from the lungs, and $10^4$-$10^5$
CFU from the trachea of TLR4<sup>d</sup> mice on day 3 post-inoculation (Fig. 2B). Serum antibodies are effective in clearing similar bacterial burdens from the lower respiratory tract of wild type mice (Fig. 2A). However, transfer of serum had no measurable effect on the bacterial numbers on day 3 post-inoculation in TLR4<sup>d</sup> mice (Fig. 2B) indicating TLR4 is required for the in vivo antimicrobial effects.

**TLR4 is required for the early neutrophil response in the lungs**

TLR4<sup>d</sup> mice have previously been shown to be defective in inducing various pro-inflammatory cytokines in response to gram-negative bacteria (2). One of the possible reasons for the inability of serum antibodies to clear bacteria from TLR4<sup>d</sup> mice may be attenuated inflammatory cell recruitment as a result of a dysregulated chemokine response. To determine if TLR4 is required for the recruitment of inflammatory cells to the site of infection we inoculated TLR4<sup>s</sup> and TLR4<sup>d</sup> mice with *B. bronchiseptica* or a PBS control. Mice were sacrificed 12 hours post-inoculation and lung leukocytes were enumerated. The total lung leukocytes in TLR4<sup>s</sup> mice were twice that of TLR4<sup>d</sup> mice (Fig. 3). No significant difference in the number of macrophages or lymphocytes was observed, however the early neutrophil response was essentially absent in TLR4<sup>d</sup> mice (Fig. 3). These results suggest that the failure of TLR4<sup>d</sup> mice to clear bacteria upon adoptive transfer of antibodies may be due to defective neutrophil recruitment.

**Neutrophils are required for antibody-mediated bacterial clearance**

Previous reports have suggested the early inflammatory response to *B. bronchiseptica* is characterized by a rapid infiltration of polymorphonuclear cells (PMNs) to the site of infection (16). However, TLR4<sup>d</sup> mice show a defective early PMN response, raising the possibility that PMNs are critical in antibody-mediated clearance of
bacteria. In order to investigate the involvement of PMNs, we used mAb RB6-8C5, which has been shown to deplete granulocytes without affecting circulating and resident macrophages or dendritic cells (6). Groups of wild type mice were intraperitoneally injected with either PBS or 1mg of RB6-8C5 mAb, which has been previously used to deplete PMNs in vivo (33). Subsequently these mice were inoculated with *B. bronchiseptica* and adoptively transferred either naive or convalescent-phase serum as described previously. On day 3 post-inoculation, mice that were treated with RB6-8C5 and naive serum harbored approximately $10^7$ CFU in the lungs, and $10^5$ CFU in the trachea (Fig. 4). These data indicate that treating mice with RB6-8C5 did not significantly alter bacterial colonization of the respiratory tract. As expected, mice treated with PBS and convalescent-phase serum cleared the bacteria from the trachea and lungs by day 3 post-inoculation. In contrast, mice treated with convalescent-phase serum and RB6-8C5 harbored 10,000 fold more CFU in the trachea and 1,000,000 fold higher CFU in the lungs than mice treated with convalescent-phase serum and PBS on day 3 post-inoculation (Fig. 4). Treatment with RB6-8C5 did not significantly alter the recruitment of macrophages or lymphocytes into the lungs of infected mice (data not shown). To rule out the possibility that treating mice with RB6-8C5 affected complement, we injected RB6-8C5 3 days before inoculation. Results obtained by pre-treating mice with RB6-8C5 were not significantly different from treating on the same day of inoculation and these mice also failed to clear bacteria from the lower respiratory tract (data not shown). These data suggest that neutrophils are required for antibody-mediated clearance of bacteria.

**FeγRs are required for antibody-mediated bacterial clearance in the lungs.**
Neutrophils are known to clear extracellular bacteria by phagocytosis via various receptor-dependent mechanisms (13). Fc receptors present on neutrophils can specifically recognize antibody bound bacteria and facilitate phagocytosis (13). The requirement for FcγRs in antibody-mediated clearance was investigated using mice which lack all three FcγRs (FcγR−/−) (30). Groups of wild type C57BL/6 and FcγR−/− mice were inoculated with *B. bronchiseptica* and adoptively transferred naïve or convalescent-phase serum as described previously. As before, convalescent-phase serum, but not naïve serum, dramatically reduced bacterial numbers on day 1 and cleared the bacteria by day 3 post-inoculation in the trachea and lungs of wild type mice (Fig. 5A). The number of bacteria recovered from the lungs of FcγR−/− mice treated with convalescent-phase serum was significantly higher than that of wild type mice treated similarly on both days 1 (100 fold) and 3 (100,000) post-inoculation (Fig. 5A), indicating FcγRs are required for antibody-mediated bacterial clearance in the lungs. Interestingly, the number of bacteria recovered from the tracheas of convalescent-phase serum treated FcγR−/− mice was significantly lower than that recovered from naïve serum treated wild type or FcγR−/− mice (Fig. 5A). These data indicate that FcγRs are required for antibody-mediated clearance of bacteria in the lungs but not in the trachea.

To further determine if any individual FcγR is critical for the antibody-mediated clearance of bacteria, combinations of mouse strains which lack one or more FcγRs were used. These include FcγRII−/−, FcγRIII−/−, and γ common chain deleted mice that lack FcγRI and FcγRIII (FcγRI−/− & III−/−) (35, 36). These mice were inoculated with the standard dose of bacteria and adoptively transferred naïve or convalescent-phase serum as previously described. Mice lacking all three FcγRs had significantly higher bacterial
numbers in the lungs than did mice lacking one or two FcγRs, which rapidly cleared bacteria from the lungs upon transfer of convalescent-phase serum (Fig. 5B). These data indicate that no specific FcγR is required and suggest that the presence of any single FcγR is sufficient for antibody-mediated bacterial clearance in lungs.

The inability of FcγR−/− mice to rapidly eliminate bacteria upon adoptive transfer of antibodies may be due to either the absence of FcγR bearing cells at the site of infection or the failure of these cells to phagocytose opsonized bacteria. In order to differentiate between these two possibilities, we analyzed the inflammatory cell recruitment (neutrophils, macrophages and lymphocytes) in the lungs of FcγR−/− mice in response to bacterial infection. There was no significant difference between the total number of inflammatory cells or individual cell populations recovered from the lungs of wild type and FcγR−/− mice (Fig. 6), suggesting no impairment of cellular recruitment in FcγR−/− mice. Furthermore, histopathology of lungs from wild type and FcγR−/− mice infected with *B. bronchiseptica* did not reveal a significant difference in inflammation or cellular lesions (data not shown). These results suggest that the failure of FcγR−/− mice to clear bacteria upon adoptive transfer of antibodies is not due to a defect in recruitment of phagocytic cells but may be due to the impaired phagocytosis of opsonised bacteria.

**Complement and CR3 are required for antibody-mediated bacterial clearance in the lower respiratory tract.**

Complement can augment the effect of antibody-mediated bacterial clearance in vivo by enhancing phagocytosis via FcγRs or by direct bacterial lysis (19). Antibodies bound to bacteria can activate complement resulting in down stream effects such as lysis via membrane attack complex (MAC), release of chemoattractants C3a and C5a, and
iC3b-mediated opsonization via CR3 (7, 37, and 38). Since the combination of antibody and complement-mediated opsonization can synergistically increase phagocytosis, we evaluated the role of complement using mice that lack C3, the central complement component required for both classical and alternate activation pathways. Groups of wild type (C57BL/6) and C3⁻/⁻ mice were inoculated and adoptively transferred either naïve or convalescent-phase serum as previously described. Recovery of bacteria from these mice was analyzed on days 1 and 3 post-inoculation in the respiratory organs. Wild type mice treated with convalescent-phase serum rapidly reduced the bacterial numbers in the trachea and lungs by day 1 and completely cleared the bacteria by day 3 post-inoculation (Fig. 7A). However, C3⁻/⁻ mice failed to reduce the bacterial numbers from the trachea and lungs on days 1 and 3 post-inoculation and harbored 1,000 to 100,000 fold higher number of bacteria than wild type mice (Fig. 7A). These results indicate that C3 is necessary for the antibody-mediated clearance of bacteria. These data along with data from FcγR⁻/⁻ mice suggest that antibody-mediated neutralization of bacterial adhesins or toxins is not sufficient to clear the bacteria from the lower respiratory tract.

To investigate whether antibody-mediated bacterial clearance in vivo is via MAC-mediated lysis, we used mice that lack C5, which is essential for MAC formation and C5a release (19). Groups of wild type and isogenic C5⁻/⁻ mice were inoculated and adoptively transferred 200-µl of naïve or convalescent-phase serum as previously described. Both wild type and C5⁻/⁻ mice treated with convalescent, but not naïve serum, cleared bacteria from the trachea and lungs (Fig. 7B) indicating that C5-dependent mechanisms such as MAC formation and C5a release are not necessary for complement-dependent antibody-mediated bacterial clearance.
Since C3 is required for the release of chemoattractant the C3a, we investigated whether C3 is required for inflammatory cell recruitment by enumerating the lung leukocytes recovered from wild type and C3−/− mice. Wild type and C3−/− mice were inoculated with bacteria, and the number of neutrophils, macrophages, and lymphocytes were enumerated in the lungs 12 hr post-inoculation. No significant differences in the total number of cells or individual cell populations were noted between wild type and C3−/− mice (Fig. 6). Total neutrophil counts were slightly increased both in wild type and C3−/− mice after they were treated with convalescent-phase serum (Fig. 6). Furthermore, histological evaluation of lungs from wild type and C3−/− mice inoculated with bacteria showed similar levels of cellular infiltrates and inflammation (data not shown). Thus, no significant impairment in the inflammatory cell recruitment was observed in the absence of C3a and C5a in response to bacteria or transfer of serum antibodies.

Since complement appears to be required for functions other than direct lysis or cellular recruitment, we hypothesized that it may mediate complement-dependent opsonization via CR3 present on phagocytes such as neutrophils. In order to investigate the importance of CR3, wild type (C57BL/6) and CD11b−/− (CR3−/−) mice were inoculated with *B. bronchiseptica* and adoptively transferred 200-µl of either naïve serum or convalescent-phase serum. CR3−/− mice treated with naïve serum harbored similar numbers of bacteria in the various respiratory organs on day 3 post-inoculation as that of wild type mice treated similarly (Fig. 7C). However, CR3−/− mice treated with convalescent-phase serum harbored bacterial numbers that were approximately 100 fold higher in the trachea and 10,000 fold higher in the lungs when compared to similarly treated wild type mice (Fig. 7C). These data indicate that CR3 is required for efficient...
antibody-mediated bacterial clearance from the lungs and trachea. Together these data suggest that complement is required for opsonization and phagocytosis via CR3 but not for C3a and C5a dependent chemotaxis or for lysis of bacteria.
DISCUSSION

Elucidation of the immune mechanisms that eliminate pathogens from the body is essential for the design of therapeutic and prophylactic measures against various infectious diseases. Although a large body of literature is available on the role of cell-mediated and humoral immune responses and their specific mechanisms in eliminating bacteria from the systemic organs such as liver, kidneys, etc, such exhaustive work is lacking in respiratory infections. Antibodies are believed to play a major role in immunity to respiratory pathogens such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* (8, 11, and 27). However, the mechanisms behind antibody-mediated clearance of these extracellular pathogens are not completely understood. Much of the current understanding of how antibodies may facilitate clearance of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* come from in vitro studies using monoclonal antibodies (18, 32). However, the in vivo mechanisms of antibody-mediated bacterial clearance are likely be more complicated than the findings of in vitro studies imply. Here we describe the mechanisms required for the antibody-mediated clearance of the natural mouse respiratory pathogen *B. bronchiseptica* from the respiratory tract.

Previously we have shown that adoptive transfer of convalescent-phase serum rapidly clears *B. bronchiseptica* from the lungs and trachea of mice (20). Using this approach, we found that TLR4 is critical in the antibody-mediated clearance of *B. bronchiseptica*. Although, TLR4 has been implicated to play an important role in generation of an effective adaptive immune response (25), our data show that TLR4 is also critical in the function of antibodies in clearing a bacterium. We also observed that TLR4 is required for the early recruitment of PMNs to the lungs and those PMNs are necessary for
antibody-mediated clearance of bacteria. These data are consistent with a model in which TLR4 is involved in recruiting the cells, such as neutrophils, required for antibody-mediated bacterial clearance.

Our data also indicate that complement is essential for antibody-mediated clearance of *B. bronchiseptica*, although the role of membrane attack complex-mediated lysis, and C3a- and C5a-mediated cellular recruitment appears to be limited. These findings were surprising since the membrane attack complex kills this bacterium in vitro (11) and C5a is an extremely potent chemoattractant (37, 38). These data, however, do not rule out the possibility that C3a plays an important role in the activation of the recruited cells. Another downstream effect of complement cascade initiation is cleavage of C3 into iC3b, which opsonizes bacteria for enhanced phagocytosis via complement receptors such as CR3 (38). We have observed that CR3−/− mice are unable to clear *B. bronchiseptica* from the lower respiratory tract even upon transfer of convalescent-phase serum, suggesting C3 is required for iC3b mediated opsonization which facilitates antibody-mediated phagocytosis and clearance of *B. bronchiseptica*. FcγRs were also found to be essential for bacterial clearance in the lungs. However, the presence of any single FcγR is sufficient to clear opsonised bacteria. Previous reports suggest a possible interplay between FcγRs and CR3 in phagocytosis and activation (19). Together, these data suggest a model for how antibodies mediate the clearance of *B. bronchiseptica* from the lower respiratory tract of mice: *B. bronchiseptica* induced inflammation via TLR4 is critical in the initial cellular recruitment of FcγR and CR3 bearing PMNs that phagocytose antibody- and iC3b-coated bacteria. Further analysis of the interaction of
innate immune receptors, complement, and FcγRs in bacterial clearance is currently in progress.

It is widely accepted that there is a significant difference in the immune responses generated in the upper and lower respiratory tracts (41). This is reflected by the fact that the upper respiratory tract is more permissive of bacterial colonization than the lower respiratory tract (42). In our model, serum antibodies have no effect on the bacterial numbers in the nasal cavity but can rapidly clear *B. bronchiseptica* from the trachea and lungs (20). Our data further suggest that there are significant differences in the mechanism of antibody-dependent bacterial clearance within the various parts of the lower respiratory tract. FcγRs are not required for the antibody-mediated clearance of *B. bronchiseptica* from the trachea but are essential in the lungs. In addition, C3 but not C5, is required in both organs. This suggests that serum antibody initiated complement cascade results in iC3b-mediated opsonization and subsequent phagocytosis in the trachea. However, in the lungs, FcγR-mediated phagocytosis is as critical in the elimination of bacteria as CR3-mediated phagocytosis. These differences reflect different physiology in the trachea and lungs. In the absence of the powerful muco-ciliary escalator function of the trachea, the immune response in the lungs may be dependent upon phagocytosis of opsonised bacteria via FcγRs and CR3. However, the large vasculature of the lungs allows for rapid mobilization of phagocytic cells from the blood. This interesting organ-specific requirement for antibody effector function may reflect the diversity of immune mechanisms developed by hosts in order to limit the spread of pathogens to more proximal portions of the respiratory tract.
The current vaccines to many bacterial pathogens are either inactivated or subunit vaccines. These vaccines generate a potent serum antibody response and are effective in preventing disease symptoms upon infection. However, they often fail to prevent subclinical infections, providing an opportunity for these bacteria to infect an immune individual and persist in a population (9, 12, and 14), which presents an ongoing health risk to unvaccinated, immunocompromised and elderly populations. Since the objective of an ideal vaccine is not only to prevent disease but also to prevent spread of the disease, understanding the molecular mechanisms of bacterial clearance will provide a rationale to modifying the existing vaccination strategies to improve long lasting protection from infection. Our current studies delineate antibody-mediated bacterial clearance of the prototypical respiratory pathogen *B. bronchiseptica*. Since complement is required in the trachea and lungs, vaccines may be more effective if they induce complement-fixing isotypes of antibodies. Similarly, the generation of antibodies that have high affinity to FcγR may be more effective in preventing infection in the lungs. Determination of specific isotypes and their cognate antigens may greatly aid in the design of novel effective vaccines.
Fig. 1 TLR4 is required for vaccine-induced but not for infection-induced immunity. A) Mice were either inoculated or vaccinated with *B. bronchiseptica*, and 28 days later challenged with 5 $\times 10^5$ CFU of *B. bronchiseptica*. Trachea and lungs were harvested on day 3 post-challenge and bacteria recovered from these organs were enumerated. B) Sera from vaccinated TLR4$^+$ and TLR4$^d$ mice were collected on day 28 post-vaccination and anti-*Bordetella* antibody titers were determined by ELISA. C) Sera from infected and vaccinated TLR4$^+$ and TLR4$^d$ mice were collected on day 28 post-inoculation or vaccination. TLR4$^+$ mice were inoculated with 5 $\times 10^5$ CFU of *B. bronchiseptica* and adoptively transferred 200-µl of TLR4$^+$ or TLR4$^d$ convalescent or vaccine serum. The lungs and trachea were harvested on day 3 post-inoculation and bacteria recovered from these organs were enumerated.
Fig 2. TLR4 is required for function of antibodies. A) TLR4<sup>s</sup> and TLR4<sup>d</sup> mice were inoculated with 5 X10<sup>5</sup> CFU of *B. bronchiseptica* and adoptively transferred 200-µl of TLR4<sup>s</sup> convalescent-phase serum on day 0. The lungs and trachea were harvested on day 3 post-inoculation and bacteria recovered from these organs were enumerated.

B) TLR4<sup>s</sup> and TLR4<sup>d</sup> mice were inoculated with 5 X10<sup>2</sup> CFU of *B. bronchiseptica* and adoptively transferred 200-µl of TLR4<sup>s</sup> convalescent-phase serum on day 0. The lungs and trachea were harvested on day 3 post-inoculation and bacteria recovered from these organs were enumerated.
Fig. 3. TLR4 is required for the early recruitment of PMNs to the lungs. TLR4<sup>+</sup> and TLR4<sup>−/−</sup> mice were inoculated with 5 X 10<sup>5</sup> CFU of <i>B. bronchiseptica</i> and lungs were collected after 12 hrs. Lungs were digested with collagenase I and Dnase I and leukocytes were separated by density gradient and total leukocytes and individual cell types were determined.
Fig. 4 PMNs are required for antibody-mediated clearance of *B. bronchiseptica*.

Groups of mice were injected with 1 mg of RB6-8C5 mAbs or PBS. These mice were subsequently injected with 200-µl of either convalescent-phase or naïve serum. All mice were inoculated with 5 x 10⁵ CFU of *B. bronchiseptica*. The lungs and trachea were harvested on day 3 post-inoculation and bacteria recovered from these organs were enumerated.
**Fig. 5 FcγRs are necessary for antibody-mediated clearance of *B. bronchiseptica* from the lungs.** A) FcγR−/− mice were inoculated with 5 × 10^5 CFU of *B. bronchiseptica* and treated with either convalescent-phase or naïve serum on day 0. The lungs and trachea were harvested on day 1 and 3 post-inoculation and bacteria recovered from these organs were enumerated. B) Mice were inoculated with 5 × 10^5 CFU of *B. bronchiseptica* and injected with 200-µl of either convalescent-phase or naïve serum on day 0. Lungs were collected on day 3 post-inoculation to assess the bacterial numbers.
Fig. 6. C3<sup>−/−</sup> and FcγR<sup>−/−</sup> mice show normal leukocyte recruitment to the lungs in response to <i>B. bronchiseptica</i>. WT, C3<sup>−/−</sup> and FcγR<sup>−/−</sup> mice were inoculated with <i>B. bronchiseptica</i> and lungs were collected after 12 hrs. Lungs were digested with collagenase I and Dnase I and leukocytes were separated by density gradient and total leukocytes and individual cell types were determined.
Fig. 7 Complement component C3, and CR3 are necessary for antibody-mediated clearance of *B. bronchiseptica*. A) WT and C3<sup>−/−</sup> mice were inoculated with 5 X 10<sup>5</sup> CFU of *B. bronchiseptica* and treated with 200-µl of either convalescent-phase or naïve serum on day 0. The lungs and trachea were harvested on day 1 and 3 post-inoculation and bacteria recovered from these organs were enumerated. B) WT and C3<sup>−/−</sup> mice were inoculated with 5 X 10<sup>5</sup> CFU of *B. bronchiseptica* and treated with 200-µl of either convalescent-phase or naïve serum on day 0. The lungs and trachea were harvested on day 3 post-inoculation and bacteria recovered from these organs were enumerated. C) WT and CR3<sup>−/−</sup> mice were inoculated with 5 X 10<sup>5</sup> CFU of *B. bronchiseptica* and treated with 200-µl of either convalescent-phase or naïve serum on day 0. The lungs and trachea were harvested on day 3 post-inoculation and bacteria recovered from these organs were enumerated.
REFERENCES


    Wiener-Kronish.** 2004. TLR4 signaling is essential for survival in acute lung 
    injury induced by virulent *Pseudomonas aeruginosa* secreting type III secretory 

    locally delivered polyclonal immunoglobulin against *Pseudomonas aeruginosa* 

    **70**:1-14.

    Neutrophils from term and preterm newborn infants express the high affinity 


15. **Garcia-Suarez Mdel, M., M. D. Cima-Cabal, N. Florez, P. Garcia, R. 
    Cernuda-Cernuda, A. Astudillo, F. Vazquez, J. R. De los Toyos, and F. J. 
    Mendez.** 2004. Protection against pneumococcal pneumonia in mice by 


22. **Mann, P. B., K. D. Elder, M. J. Kennett, and E. T. Harvill.** 2004. Toll-like receptor 4-dependent early elicited tumor necrosis factor alpha expression is


Achtman, R. Atkin, S. Baker, D. Basham, N. Bason, I. Cherevach, T.
Chillingworth, M. Collins, A. Cronin, P. Davis, J. Doggett, T. Feltwell, A.
Goble, N. Hamlin, H. Hauser, S. Holroyd, K. Jagels, S. Leather, S. Moule, H.
Sanders, D. Saunders, K. Seeger, S. Sharp, M. Simmonds, J. Skelton, R.
Squares, S. Squares, K. Stevens, L. Unwin, S. Whitehead, B. G. Barrell, and
D. J. Maskell. 2003. Comparative analysis of the genome sequences of
Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Nat

29. Qureshi, S. T., and R. Medzhitov. 2003. Toll-like receptors and their role in
experimental models of microbial infection. Genes Immun 4:87-94.


Tomibe. 1984. Protection against infection with Pseudomonas aeruginosa by
passive transfer of monoclonal antibodies to lipopolysaccharides and outer

Kaufmann. 2000. Rapid neutrophil response controls fast-replicating intracellular
bacteria but not slow-replicating Mycobacterium tuberculosis. J Infect Dis
181:671-80.


CHAPTER 5
PERTUSSIS TOXIN INHIBITS NEUTROPHIL RECRUITMENT TO DELAY ANTIBODY-MEDIATED CLEARANCE OF BORDETELLA PERTUSSIS.
ABSTRACT

Infectious diseases with high transmission rates and long lasting immunity result in disease primarily in children and immune adults. Under these conditions there is strong selection for the ability to reinfect immune hosts. Although whooping cough is considered a childhood disease, there is substantial evidence that children are infected by asymptomatic adult carriers, and there are increasing numbers of adult cases of *Bordetella pertussis* disease. In order to improve the control of this and other such diseases it is critical to understand how *B. pertussis* is able to remain endemic even in highly vaccinated or immune populations. To this end we have examined the nature of sterilizing immunity to *B. pertussis*. Antibodies are necessary to control infection but do not rapidly clear *B. pertussis* from the lower respiratory tract. However, antibodies affect *B. pertussis* after a delay of at least a week, by a mechanism that involve Fc receptors and is dependent on the recruitment of neutrophils. The kinetics of neutrophil recruitment correlate with that of antibody-mediated clearance, and Pertussis Toxin, has been shown to delay neutrophil recruitment. A PTX mutant induced much more rapid neutrophil recruitment and antibodies efficiently cleared this strain from the lower respiratory tract within 3 days. Depletion of neutrophils abrogated the effects of anti-*B. pertussis* antibodies on the PTX mutant. Together these results indicate that *B. pertussis* avoids rapid antibody-mediated clearance by PTX-mediated inhibition of neutrophil recruitment to the lungs.
INTRODUCTION

Widespread use of vaccines in developed nations has decreased the incidence of whooping cough (12). However, recent epidemiological surveys reveal that a majority of individuals in a vaccinated population are transiently infected with the causative agent, *Bordetella pertussis*, and that it is widespread and endemic (20). In vaccinated populations the bacterium usually subclinically infects immunized individuals who serve as the primary host (42). Although disease may be the greatest public health concern it may not be critical to the spread of *B. pertussis* within vaccinated populations. In fact, childhood disease predates extensive mixing of children and appears to commonly have as its source an adult, nonsymptomatic, carrier (41). The ability of *B. pertussis* to circulate in vaccinated and immune populations has been known clinically for years but has not been well studied experimentally. Although experimental infection of naïve mice may simulate disease, infection of vaccinated or convalescent animals with waning immunity may be more relevant to the biology of the bacterium in a vaccinated population.

Current pertussis vaccines induce a strong serum antibody response which has been shown to be critical for protection from the disease (19, 24, 25, and 36). Interestingly, the bacterium is able to infect humans with high serum antibody titers to *B. pertussis* antigens (33), suggesting the bacterium has mechanism/s to resist the effects of antibodies. Using animal models, we and others have previously shown that although B cells are necessary for *B. pertussis* clearance from the respiratory tract (19, 24, and 25), adoptively transferred serum antibodies have no effect on bacterial numbers for the first 7 days post-inoculation (19, 24), but begin to control and clear the bacteria thereafter. The ability to resist rapid antibody-mediated clearance should increase the duration and intensity of infection, both of which
facilitate the transmission of the bacteria and would therefore be critical to the endemism of *B. pertussis* in vaccinated populations.

*B. pertussis* is thought to have emerged from a *B. bronchiseptica*-like progenitor (40, 29). These closely related subspecies share a similar set of virulence determinants but a different host range (30). Interestingly, while both require B cells for their clearance from the respiratory tract, only *B. bronchiseptica* is rapidly (within 3 days) cleared by adoptively transferred serum antibodies (19). We previously elucidated the mechanism of antibody-mediated clearance of *B. bronchiseptica* in order to determine the pathway that is presumably inhibited by *B. pertussis* (Chapter 4). Serum antibody-mediated clearance of *B. bronchiseptica* requires a TLR4-induced early recruitment of neutrophils that phagocytose bacteria via FcγRs and CR3. We hypothesize that serum antibody-mediated clearance of *B. pertussis* also requires neutrophils and that it may resist rapid serum antibody-mediated clearance by inhibiting neutrophil recruitment. Interestingly, Carbonetti et al have shown that pertussis toxin (PTX) facilitates *B. pertussis* colonization by altering neutrophil recruitment (6). Hence, we sought to determine the role of PTX in delaying antibody-mediated clearance of *B. pertussis*.

PTX, which is only expressed by *B. pertussis*, is an A-B type toxin known to inhibit G-protein signaling pathways that involve Giα, interfering with a class of receptor that includes chemokine receptors (27, 28). Various in vitro studies have demonstrated its ability to inhibit the chemotaxis of neutrophils, lymphocytes and macrophages (37). Although, PTX has been implicated in the pathogenesis of whooping cough (15), its exact role in vivo is not understood. In the current analysis of the mechanism of antibody-mediated clearance, we observed that *B. pertussis* clearance is similar to that of *B. bronchiseptica*, except for delayed
kinetics that correlated with delayed recruitment of neutrophils that are required for clearance of both. PTX, which is only expressed by *B. pertussis*, inhibits rapid antibody-mediated bacterial clearance by inhibiting neutrophil recruitment to the lungs. Expression of PTX may be an adaptation strategy of *B. pertussis* to cause an acute infection and extend its infectious period in immune hosts facilitating its persistence in immune human populations.
MATERIALS AND METHODS

Bacteria

*B. pertussis* strain 536 is a streptomycin resistant derivative of Tohama I (13). 

*B. pertussis Δptx* is a PTX mutant of BP536 and is a kind gift from Drussila Burns (14). 

Bacteria were maintained on Bordet-Gengou (BG) agar (Difco) supplemented with 7.5% defibrinated sheep’s blood (Remel or Hema Resources) and 20 µg/ml streptomycin. 

Bacteria were grown in Stainer-Scholte (SS) broth with supplements and 20 µg/ml streptomycin to mid-log phase (optical densities of approximately 0.3 at 600 nm) at 37°C on a roller drum for experiments.

Mice

C57BL/6 mice were obtained from Jackson Laboratories and C3−/− mice, back-crossed extensively onto a C57BL/6 background, have been described elsewhere and were kind gifts of Dr. Rick Wetsel (8). FCγR−/− (C57BL/6 background) were obtained from Taconic and are described elsewhere (32).

Inoculation, and adoptive transfer protocols

Mice were lightly sedated with isoflurane (IsoFlo-Abbott Laboratories), and 5 X 10⁵ CFU of bacteria in 50 µl of PBS were inoculated onto the tip of the external nares. Colonization levels were determined by homogenizing the lungs in 1 X PBS and plating aliquots for colony counts. The homogenates and necessary dilutions were plated in 50 µl volumes onto BG agar with streptomycin. Colonies were counted after 3 days incubation at 37°C. For adoptive transfer experiments, intraperitoneal injection of 200µl of convalescent-phase serum obtained on day 28 post-inoculation from mice which were inoculated with BP 536, into naïve mice was immediately followed by inoculation as
described above. Animals were sacrificed on the indicated day post transfer and inoculation and colonization determined as described. Animals were handled in accordance with institutional guidelines. Statistical significance of data points was determined using a student's unpaired t-test.

**Enumeration of leukocytes in the lungs**

Total leukocytes were isolated from the lungs after collagenase and DNAse I digestion as described previously (16). Briefly, lungs were perfused with PBS and finely sheared by pair of scissors. This lung homogenate was subjected to collagenase type I and Dnase I treatment for about 3 hrs. The enzymatically treated homogenate was laid over Histopaque 1119 (Sigma Aldrich, MO) and centrifuged for 30 min at 3000 rpm. Leukocyte portion was collected and the total number of cells was determined by hemocytometer. Individual cell types were determined by staining the isolated cells with modified Giemsa stain by a certified clinical laboratory technician.

**Estimation of cytokines and chemokines**

MH-S cells, murine alveolar macrophage cell line (26), were obtained from ATCC and cultured in DMEM medium supplemented with 10% FBS. Cells were grown to confluency and incubated with either *B. pertussis* or *B. pertussisΔptx* at MOI of 1:10 for 12 hr at 37°C. Culture supernatants were collected and filter sterilized and stored at -80°C for further use. Amount of TNFα, IL-1β, IL-6, IFNγ, IL-10, KC, JE, MIP1α, and MIP2 were estimated by standard ELISA.

**Neutrophil migration assay**

10^5 primary murine aortic endothelial cells were cultured on 3μM transwells with DMEM medium for 24hrs to simulate blood vessel wall. Peripheral PMNs were collected
from C57BL/6 mice by differential density separation using Histopaque 1119 and 1077 (17). The percentage of PMNs was estimated by Giemsa staining of isolated cells. 10^5 PMNs were layered on the endothelial cells in transwells. 200\mu l of supernatant from macrophages cultured with \textit{B. pertussis} or \textit{B. pertussis} \textit{Δptx} for 12 hrs was used as a source of chemoattractants in the lower well of the transwell system. After 12 hrs, the number of neutrophils migrated to the lower chamber is measured by observing 10 random fields.

\textbf{Neutrophil depletion}

RB6-8C5 is a mAb raised against Ly-6 present on neutrophils and is a kind gift from Garry Huffnagle (21). 1 mg of this mAb was injected intraperitoneally which is previously shown to deplete neutrophils for 7-14 days (34). Peripheral PMNs were enumerated to check the efficacy of the treatment.
RESULTS

Serum antibodies do not rapidly clear *B. pertussis*

We and others have previously shown that B cells are necessary for the clearance of *B. pertussis* from the lungs (19). Therefore we sought to investigate the role of serum antibodies in bacterial clearance. Groups of wild type mice were inoculated with 5 X 10^5 CFU of *B. pertussis* in 50µl of PBS by intranasal route. This inoculation regimen has been previously shown to deliver a significant number of bacteria into the lungs. Immediately after inoculation, 200µl of either naïve or convalescent-phase serum were injected by intraperitoneal route. Mice were sacrificed on days 1, 3, 7, 10 and 14 post-inoculation and lungs were harvested to enumerate the number of bacteria. Numbers of CFU recovered from the lungs of either naïve serum or convalescent-phase serum treated mice did not differ significantly on days 1, 3, and 7 post-inoculation suggesting serum antibodies have minimal effect on bacterial clearance during the first 7 days post-inoculation (Fig. 1). However, convalescent-phase serum treated mice harbored only ~1000 bacteria while naïve serum treated mice harbored ~100,000 bacteria on day 10 post-inoculation. Similarly, no bacteria could be detected in the lungs of convalescent-phase serum treated mice on day 14 post-inoculation while approximately 10^5 CFU of *B. pertussis* were recovered from the lungs of naïve serum treated mice. These data suggest that serum antibodies are able to clear *B. pertussis* from the lungs only after 1 week of infection. Apparently *B. pertussis* resists the effect of antibodies during the first week of infection, perhaps by inhibiting one or more antibody effector functions.

**FcγRs but not C3 are required for serum antibody-mediated clearance of *B. pertussis***

Antibodies may clear bacteria by neutralization, complement-mediated lysis, or opsonization for FcγR-mediated phagocytosis. We have previously shown serum antibodies
rapidly clear B. bronchiseptica from the lungs of mice and the mechanism involved both complement and FcγRs (Chapter 4). Since B. pertussis is very closely related to B. bronchiseptica but is cleared much more slowly by serum antibodies, we predicted that B. pertussis has some mechanism to resist one or more of these antibody effector functions early in the infection. In order to test the importance of complement in serum antibody-mediated bacterial clearance we used mice lacking the central complement component C3 required for both classical and alternate complement cascades. Groups of wild type C57BL/6 and congenic C3-/- mice were inoculated with B. pertussis as described above. Immediately after inoculation, 200-µl of naïve or B. pertussis-induced convalescent-phase serum was injected by i.p. route. 14 days after inoculation, mice were sacrificed and lungs were harvested to enumerate B. pertussis CFU. Naïve serum had no effect on bacterial numbers in the lungs of wild type or C3-/- mice (Fig. 2A). Convalescent-phase serum completely cleared the bacteria from the lungs of wild type and C3-/- mice by 14 days. These data indicate that C3 is not required for serum antibody-mediated clearance of B. pertussis.

In order to test the importance of FcγRs in antibody-mediated clearance of B. pertussis, we used FcγR-/- mice, which lack all three Fc receptors for IgG and one for IgE. Groups of FcγR-/- mice were adoptively transferred naïve serum, or convalescent-phase serum and inoculated with B. pertussis as described previously. As opposed to wild type mice treated with convalescent-phase serum, which completely cleared the bacteria from the lungs within 14 days, FcγR-/- mice treated similarly had no significant reduction in bacterial numbers from the lungs (Fig. 2B). These data suggest that FcγRs are required for the serum antibody-mediated clearance of B. pertussis. The above data suggest that antibodies are not functioning
by neutralization alone or via complement-mediated lysis and establish that antibodies clear *B. pertussis* via phagocytosis by FcγR bearing cells that are recruited into the lungs.

**Neutrophils are required for antibody-mediated clearance of *B. pertussis***

We have previously shown that FcγR-bearing neutrophils are the primary inflammatory cells recruited to the lungs following infection with *B. bronchiseptica* (13) and are required for rapid antibody-mediated bacterial clearance. Since *B. pertussis* clearance required FcγRs, we sought to determine if neutrophils are required for antibody-mediated bacterial clearance. Groups of C57BL/6 mice were intraperitoneally injected with PBS or 1 mg of anti-Ly-6 monoclonal antibodies (RB6-8C5). This treatment has been previously shown to deplete neutrophils for at least 2 weeks with no apparent effect on other cells such as macrophages or dendritic cells (9, 34). Following treatment, mice were inoculated with *B. pertussis* and adoptively transferred naïve or immune serum as described above. The mice were sacrificed on day 14 post-inoculation and bacterial numbers in the lungs were enumerated. Naïve serum had no effect on the bacterial numbers in the lungs of mice treated with PBS or RB6-8C5. However, immune serum completely cleared bacteria from the lungs of mice treated with PBS but not RB6-8C5. These results indicate that the neutrophils recruited to the lungs are required for antibody-mediated bacterial clearance.

**Pertussis toxin inhibits antibody-mediated clearance of *B. pertussis***

*B. bronchiseptica* and *B. pertussis* are very closely related and share a majority of the known virulence genes (29, 40). Both bacteria require similar antibody effector functions and phagocytic cells for their clearance from the lungs of mice (Fig. 2 and Chapter 4). Yet, immune serum begins to reduce the numbers of *B. bronchiseptica* as early as day 1 whereas it has no effect on the number of *B. pertussis* for first 7 days post-inoculation (19). These data suggested

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that some *B. pertussis* specific virulence gene(s) may play a role in delaying antibody-mediated bacterial clearance. Pertussis toxin (PTX) is a *B. pertussis*-specific virulence factor that has been extensively studied in vitro and shown to inhibit the downstream effects of G-protein coupled receptors including that of many chemokine receptors (27, 28). One plausible explanation for the delayed bacterial clearance may be that secreted PTX inhibits early neutrophils recruitment which is necessary for antibody-mediated clearance. In support of this argument, we have previously observed that *B. bronchiseptica* induces significantly more neutrophil infiltration than *B. pertussis* on day 3 post-inoculation (13). Therefore, we predicted that in the absence of PTX, *B. pertussis* may be more susceptible to antibody-mediated clearance. In order to test this hypothesis, we inoculated mice with $5 \times 10^5$ CFU of *B. pertussis* or *B. pertussis* $\Delta ptx$ as described previously and adoptively transferred either naïve or convalescent-phase serum. Lungs were harvested from these mice on days 3 and 7 post-inoculation and bacterial numbers in the lungs were enumerated. In the lungs of naïve serum treated mice, the numbers of wild type *B. pertussis* recovered were 100 fold more than the numbers of *B. pertussis* $\Delta ptx$ (Fig. 4). These data are consistent with the findings by Carbonetti et al that pertussis toxin is required for efficient colonization by *B. pertussis* (6). Interestingly, convalescent-phase serum rapidly reduced numbers of *B. pertussis* $\Delta ptx$ in the lungs as early as 3 days post-inoculation and completely cleared this bacterium within 7 days while it had no effect on the numbers of *B. pertussis*. These data suggest that PTX is involved in inhibiting antibody-mediated clearance of *B. pertussis* perhaps by inhibiting neutrophils migration to the lungs.

**Pertussis toxin reduces neutrophil recruitment to the lungs**
*B. bronchiseptica* induces significantly higher numbers of neutrophils than *B. pertussis* during the first week of infection and is rapidly cleared by serum antibodies (13, 19, and 31). Since neutrophils are required for antibody-mediated clearance of *B. pertussis*, we predicted *B. pertussis* has mechanisms to inhibit neutrophil recruitment to the lungs to resist the effect of serum antibodies. Since previous studies have shown that PTX reduces the proportion of neutrophils recovered in the bronchoalveolar lavage (BAL) fluid (6) we hypothesized that PTX inhibits neutrophil migration to the lungs, decreasing their numbers and thereby inhibiting antibody-mediated bacterial clearance. To test this, groups of mice were inoculated with *B. pertussis* or *B. pertussisΔptx* as described previously and leukocytes recruited in the lungs were enumerated. Approximately 5 to 6 million leukocytes were observed on day 1 post-inoculation, and 7-8 million on days 3 and 7 in the lungs of mice inoculated with *B. pertussis* (Fig. 5). In contrast, lungs of mice inoculated with *B. pertussisΔptx* harbored approximately 10-11 million leukocytes on day 1, 12 million on day 3 and 10 million on day 7 post-inoculation. The majority of leukocytes in the lungs of mice inoculated with *B. pertussis* were macrophages (~4 millions) while a small percentage of cells (~2 million) were lymphocytes and the rest were neutrophils (~1 million) on day 1 post-inoculation. However, the majority of leukocytes in the lungs of mice inoculated with *B. pertussisΔptx* were neutrophils (~6 million). A similar pattern was also observed on days 3 and 7, when the majority of the leukocytes recruited to the lungs of mice inoculated with *B. pertussisΔptx* were neutrophils while macrophages predominated in the lungs of mice inoculated with *B. pertussis*. However, the absolute number of macrophages and lymphocytes recruited were similar in the two groups. These data indicate that the higher proportion of neutrophils previously reported to be recruited by *B. pertussisΔptx* can be attributed to a large increase in absolute numbers of
neutrophils within the lungs. Thus, it can be concluded that PTX inhibits neutrophil recruitment to the lungs and inhibits antibody-mediated clearance of *B. pertussis*. On day 10, however, the lungs of mice inoculated with *B. pertussis* harbored approximately 10 million leukocytes of which 5-6 million cells were neutrophils and 2-3 millions were lymphocytes, indicating that the effects of PTX are ultimately overcome, and neutrophils are recruited to the lungs later during infection correlating with the time frame in which antibodies are effective against *B. pertussis*.

**Antibody-mediated clearance of *B. pertussisΔptx* requires FcγRs and neutrophils**

The above data indicate a strong correlation between neutrophil recruitment and serum antibody-mediated bacterial clearance suggesting opsonization via FcγRs of neutrophils as the mechanism of *B. pertussis* clearance. However, it is possible that *B. pertussisΔptx* may be cleared by serum antibodies by a mechanism that is different from that of wild type *B. pertussis*. Therefore, we determined the mechanism of antibody-mediated clearance of *B. pertussisΔptx*. Groups of C57BL/6 and FcγR−/− mice were inoculated with *B. pertussisΔptx* and adoptively transferred 200μl of either naïve or immune serum as described above. Naïve serum had no significant effect on the bacterial numbers in the lungs of wild type and FcγR−/− mice on either days 3 or 7 (Fig. 6A). Immune serum rapidly reduced bacterial numbers in the lungs of wild type mice by day 3 post-inoculation and completely cleared bacteria by day 7. However, no significant reduction in the numbers of bacteria was observed in the lungs of immune serum treated FcγR−/− mice on days 3 and 7 post-inoculation, indicating FcγRs are required for antibody-mediated clearance of *B. pertussisΔptx*.

We predicted, based on above data, that neutrophils are involved in rapid clearance of *B. pertussisΔptx*. Groups of C57BL/6 mice were inoculated with *B. pertussisΔptx* and adoptively
transferred 200µl of either naïve or immune serum as described previously. Half of them were also injected with 1 mg of RB6-8C5 to deplete neutrophils and other half with PBS as control. The lungs of naive serum and RB6-8C5 treated mice harbored approximately 10-100 fold higher numbers of bacteria than the lungs of naïve serum and PBS treated mice (Fig. 6B). These data strongly suggest that PTX enables *B. pertussis* to colonize lung by inhibiting neutrophil recruitment, as proposed by Carbonetti et al (6). Immune serum rapidly reduced the number of *B. pertussis Δptx* in the lungs of mice treated with PBS on day 3 and completely cleared bacteria on day 7 post-inoculation. However, immune serum failed to reduce the number of bacteria on days 3 and 7 post-inoculation in mice treated with RB6-8C5, indicating neutrophils are required for rapid serum antibody-mediated clearance of *B. pertussis Δptx*. These data indicate serum antibodies clear both *B. pertussis* and *B. pertussis Δptx* by a similar mechanism of FcγR-dependent phagocytosis by neutrophils and suggest *B. pertussis* resists rapid clearance by inhibiting neutrophils recruitment to the lungs.

**Pertussis toxin does not modulate chemokine and cytokine production by alveolar macrophages**

PTX could affect neutrophil recruitment in various ways. It is known to influence chemokine and cytokine responses (2), interfere with G protein coupled chemokine receptor signaling (1), and down regulate adhesion molecule expression on endothelial cells (35). To investigate if PTX modulates chemokine expression by alveolar macrophages in vitro, a confluent layer of alveolar macrophage cells were incubated with *B. pertussis* or *B. pertussis Δptx* at a MOI of 1:10 for 2, 4, 6 and 12 hr. The amounts of various chemokines such as MIP1α, MCP-1, JE, and KC were analyzed by ELISA. Our data indicate that macrophages produced a significant amount of various cytokines and chemokines when infected with *B.
Pertussis or *B. pertussis*Δptx. However, no significant differences were observed in the levels of various cytokines and chemokines produced by macrophages incubated with *B. pertussis* and macrophages incubated with *B. pertussis*Δptx. These data suggest that PTX does not modulate cytokine or chemokine production by macrophages in in vitro assays.

**Pertussis toxin inhibits neutrophil migration**

In order to determine if PTX acts directly on neutrophil or endothelial cells, we used an in vitro neutrophil migration assay. Essentially, this assay allowed us to determine the number of neutrophils that migrate across endothelial cells towards a source of chemokines. The average number of neutrophils per field in the wells that had media control was approximately 35 (Fig. 8) and the number of neutrophils that migrated towards supernatant from macrophages cells incubated with *B. pertussis* was slightly higher at approximately 45 per field. However, the number of neutrophils migrated towards supernatant from macrophages incubated with *B. pertussis*Δptx was approximately 400 per field. These data indicate that supernatant from macrophages incubated with *B. pertussis*Δptx had higher levels of chemoattractants or the supernatant from macrophages incubated with *B. pertussis* had some inhibitory components. Since our previous data suggest that PTX does not modulate chemokine production by alveolar macrophages, we predicted that PTX in supernatants from macrophages incubated with *B. pertussis* may alter chemotaxis of neutrophils. In order to address this possibility, equal amounts of supernatants from cells incubated with *B. pertussis* and *B. pertussis*Δptx were mixed and used for the in vitro neutrophil migration assay. Interestingly, the number of neutrophils migrated towards this mixture was approximately 50 per field; similar to the number of neutrophils migrated towards supernatant from cells incubated with *B. pertussis*. 

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These data suggest that culture supernatants from cells incubated with *B. pertussis* had some components presumably PTX that inhibit chemotaxis of neutrophils.

Since PTX has been shown affect G-protein-coupled receptor signaling pathways that may influence chemotaxis, we hypothesized that the PTX secreted by *B. pertussis* that is present in the supernatant from macrophages incubated with *B. pertussis* may directly act on neutrophils to decrease their migration across endothelial cells. To test this, we mixed various amounts of purified PTX with the supernatant from macrophages incubated with *B. pertussis∆ptx*. Neutrophil migration towards supernatants from cells incubated with *B. pertussis∆ptx* was reduced by 90% when purified PTX was added (Fig. 8). However, adding similar amount of catalytically inactivated PTX had no adverse effect on the migration of neutrophils across endothelial cells. These data suggest that PTX inhibits the migration of neutrophils across endothelial cells and this inhibition is dependent on its enzymatic activity.

The inhibitory effect of PTX may be due to its action on endothelial cells or on neutrophils. In order to differentiate between these two possibilities, endothelial cells were pre-incubated with *B. pertussis* or *B. pertussis∆ptx* and neutrophils were allowed to migrate across them towards supernatants from macrophages incubated with *B. pertussis∆ptx*. Pre-incubation of endothelial cells with either *B. pertussis* or *B. pertussis∆ptx* did not alter neutrophil migration (data not shown). In fact, neutrophil migration assays carried out in the absence of endothelial cells showed that adding PTX to supernatant from macrophages incubated with *B. pertussis∆ptx* inhibited migration of neutrophils by 50% (data not shown). These data suggest that PTX acts directly on neutrophils and affects their migration across endothelial cells.
DISCUSSION

Acute, highly contagious, immunizing pathogens face the significant epidemiological challenge of long-term persistence within the host population. Immunity results in depletion of susceptible hosts through the course of each epidemic, thereafter host replenishment requires births or loss of immunity -- which is why pathogens that convey perfect immunity result in ‘childhood diseases’ (22). Rapid contagion, in turn, results in fast transmission among hosts, which is a short-term evolutionary benefit to the pathogen (11, 5). However, it also results in large-amplitude epidemics with intervening deep epidemic troughs; In small and medium host populations the chain of transmission will be broken in the troughs so that the pathogen will go extinct. The most relevant theoretical models for childhood infections, the so-called realistic age-structured (RAS) models (4, 18), predict an endemic threshold of around 1/2 million hosts for transmission to be sustained through the epidemic troughs of acute, immunizing infections. This prediction is closely matched by epidemiological surveillance data (3, 10). Previous theoretical studies have highlighted two key adaptations that increase the height of the epidemic troughs to allow long-term endemism within smaller host communities. These are: (1) reinfection of previously immunized hosts and adult carriers and (2) prolongation of the infectious period (5). Our study is of wide epidemiological significance in showing that \textit{B. pertussis}, through expression of PTX, slows migration of neutrophils and thereby extends the infection period (relative to \textit{B. pertussis} \textit{Δptx} strains) and allow for transient reinfection of previously immunized hosts. PTX expression may, therefore, be a key adaptation by \textit{B. pertussis} for persisting in its human host.

PTX could inhibit neutrophil recruitment by modulating chemokine or cytokine production by epithelial cells and macrophages, or directly interfering with the chemokine
receptor signaling, and/or altering adhesion molecule expression that may interfere with the
diapedesis of blood leukocytes. Our in vitro data, however, suggest that PTX may be acting
directly on neutrophils, perhaps interfering with chemokine receptor signaling, although we
can not rule out the possibility that PTX may be affecting their recruitment via multiple
mechanisms in vivo. The implication for such an inhibitory effect on neutrophil recruitment is
two fold: it allows for initial *B. pertussis* colonization and provides a means to resist the effect
of serum antibodies in the lungs. Although the former effect may be important to disease, the
latter may be more relevant to the maintenance of *B. pertussis* as an endemic pathogen.

*B. bronchiseptica* and *B. pertussis* are closely related subspecies that differ in few
virulence factors providing an excellent opportunity for determining the molecular basis for
their differential host adaptation and pathogenesis. *B. bronchiseptica* can infect and colonize
the upper respiratory tract of a wide variety of non-human mammals (13). It can chronically
persist in the infected host without overt symptoms. *B. bronchiseptica* has thus evolved into a
successful commensal/pathogen of the nasal cavity where serum antibodies have little effect on
bacterial numbers. These strategies provide the bacterium sufficient time to efficiently transmit
to other animals within a population and there would be little advantage and some risk in
acquiring a mechanism to resist clearance from the lower respiratory tract. However, *B.
pertussis* causes acute but transient infection of the lower respiratory tract with pathology that
promotes rapid spread within dense human populations (43). Under these conditions there
would be a strong advantage to resisting antibody-mediated clearance that would allow *B.
pertussis* to infect immune individuals. Interestingly, both *B. pertussis* and *B. bronchiseptica*
have the genes for synthesis and transport of PTX but only *B. pertussis* expresses them (30).
Our data indicate a critical role for PTX in infection, transmission and epidemiology of *B.*
pertussis. We speculate that B. bronchiseptica retains these genes because they may provide advantage to bacteria during rapid expansion into new host populations.

PTX has long been recognized as the necessary component of pertussis vaccines (39). Various serological surveys suggested a positive correlation between anti-PTX antibody titers and protection from the disease (38). Hence, serum antibody titers to PTX are considered the most reliable indicator of protection from whooping cough and are used as a measure of efficacy of pertussis vaccines. Interestingly, antibody titers to PTX decrease much faster than those to other B. pertussis antigens (23). Since PTX allows B. pertussis to largely avoid the effects of antibodies to other antigens, the most effective vaccination strategy to B. pertussis may involve an increased focus on the induction of a long lasting serum antibody response to PTX.
Fig. 1 Serum antibodies clear *B. pertussis* after 7 days post-inoculation. Groups of 4 each of C57BL/6 mice were inoculated with $5 \times 10^5$ CFU of *B. pertussis* and adoptively transferred with 200µl of naïve serum or convalescent-phase serum. Lungs were harvested on indicated days, bacterial numbers were enumerated and expressed as geometric mean ± SE.
Fig. 2 Adoptively transferred antibodies clear *B. pertussis* in the lungs of wild type and C3+/− but not FcgR−/− mice. Groups of 4 each of WT, C3−/− and FcgR−/− mice were inoculated with 5 × 10³ CFU of *B. pertussis* and adoptively transferred 200ml of either naïve serum (white bars) or convalescent-phase serum (black bars). On day 14 post-inoculation, mice were sacrificed, bacteria in the lungs were enumerated and expressed as geometric mean ± SE.
Fig. 3 Depletion of neutrophils abrogates antibody-mediated clearance of *B. pertussis*. Groups of 4 each of wild type mice were treated with PBS or 1 mg of neutrophil depleting mAb RB6-8C5. Mice were subsequently inoculated with 5 X 10^5 CFU of *B. pertussis* and adoptively transferred 200ml of either naïve (white bars) or convalescent-phase serum (black bars). On day 14 post-inoculation, mice were sacrificed, bacteria in the lungs were enumerated and expressed as geometric mean + SE.
Fig. 4 Serum antibodies rapidly clear *B. pertussisDptx* but not *B. pertussis*. Groups of 4 each of C57BL/6 mice were inoculated with either *B. pertussis* (B.P) or *B. pertussisDptx* (B.PDPTX). Subsequently, these mice were adoptively transferred 200ml of either naïve serum (NS) or convalescent-phase serum (IS). Mice were sacrificed on indicated days, bacterial numbers in the lungs were determined and expressed as geometric means + SE.
**Fig. 5** Pertussis toxin inhibits recruitment of neutrophils to the lungs. Groups of 4 each of C57BL/6 mice were inoculated with $5 \times 10^5$ CFU of *B. pertussis* (or *B. pertussisΔptx*). Mice were sacrificed on indicated days, lungs perfused with 5 ml of cold PBS and total leukocytes per whole lung were enumerated. Individual cell type was determined by modified giemsa staining of cells.
Fig. 6 FcγRs and neutrophils are required for serum antibody-mediated clearance of *B. pertussis*Δptx. Groups of 4 each of C57BL/6 or FcγR−/− mice (A) were inoculated with 5 x 10⁵ CFU of *B. pertussis*Δptx and adoptively transferred 200 µl of either naïve or convalescent-phase serum. (B) Groups of C57BL/6 mice also received either PBS or 1 mg of mAb RB6-8C5. Lungs were harvested on indicated days, bacteria were enumerated and expressed as mean ± SE.
Fig. 7 Pertussis toxin does not modulate chemokine production by alveolar macrophages. MH-S cells were incubated with *B. pertussis* or *B.pertussis∆ptx* at MOI of 1:10 for 12hr. Culture supernatant were collected and analyzed for chemokines by ELISA and are expressed as average ± SE.
**Fig. 8 Neutrophil migration assay.** In 3mM transwell chambers, $10^5$ PMNs were allowed to migrate across endothelial cells towards supernatant from alveolar macrophages infected with *B. pertussis* or *B. pertussisDptx*. Purified pertussis toxin (PTX) or catalytically inactive pertussis toxin (DPTX) were added to the supernatant as indicated. The number of neutrophils migrated to the lower chamber were enumerated by observing 10 fields and expressed as average and SE. The experiments were repeated at least thrice.
REFERENCES


murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA. Immunopharmacology 42:135-49.


CHAPTER 6

T CELLS ARE REQUIRED FOR ADOPTIVELY TRANSFERRED SERUM ANTIBODY-MEDIATED CLEARANCE OF B. PERTUSSIS
INTRODUCTION

*B. pertussis* causes severe disease characterized by paroxysmal, or whooping cough, among young children and the elderly (8). While *B. pertussis* is responsible for the deaths of more than 600,000 children every year in developing countries (9), the mortality in developed countries is significantly lower due to widespread vaccination (18). However, the majority of vaccinated individuals have reportedly been infected with *B. pertussis* at least once without displaying clinical symptoms (4). These subclinical infections pose a potential health risk for unvaccinated, elderly, and immunocompromised individuals, among whom the incidence of whooping cough is on the rise (5). This could be attributed to the ability of bacteria to infect an immune individual without causing severe disease, and persist and circulate within an immune population (20). Recent evidence shows that the rate of whooping cough is increasing even among vaccinated children, reflecting the need for better vaccination strategies (12).

The current pertussis vaccines induce a robust serum antibody response in children (16). However, the role of antibodies in *B. pertussis* clearance remains controversial in spite of a positive correlation observed between serum antibody titers and protection from disease (4). We and others have shown that B cell deficient mice do not clear *B. pertussis* from the respiratory tract (13, 14) suggesting antibodies are required for clearance of this organism (10). However, adoptive transfer of serum antibodies from either vaccinated or convalescent animals did not confer protection against a challenge with *B. pertussis* for at least 7 days post-infection, leading multiple groups to conclude that serum antibodies have little role in bacterial clearance (13, 14). Interestingly, IgA deficient mice are normal in the clearance of *B. pertussis* indicating mucosal IgA is not
necessary for *B. pertussis* clearance (Chapter 3). Recently, we have observed that serum antibodies can clear *B. pertussis* from the respiratory tract of wild type mice only after 7 days post-infection via FcγR-mediated phagocytosis by neutrophils (Chapter 5) highlighting the importance of serum antibodies in immunity to this bacterium. Furthermore, we have demonstrated that pertussis toxin inhibits recruitment of neutrophils and thereby delays antibody-mediated *B. pertussis* clearance (Chapter 5). Thus high rates of subclinical infections observed in vaccinated human populations in spite of high serum antibody titers could be due to expression of pertussis toxin (7, 12).

Serum antibodies begin to eliminate *B. pertussis* from the reparatory tract only after 7 days post-inoculation, when a T cell response begins to be detectable (15). Consistent with this timeline, our data indicate that some B cell independent adaptive immune response is required for this serum antibody-mediated clearance of *B. pertussis*, suggesting that T cells are involved in antibody-mediated bacterial clearance. These data suggest that the T cell response is able to augment the antibody-mediated clearance by overcoming the pertussis toxin-mediated blocking/inhibition of antibody functions. Consistent with this model, MuMT mice, in the presence of adoptively transferred serum antibodies, are able to rapidly clear *B. pertussis* once a T cell response is generated but TCRα⁻ mice are not. Our data also show that CD4⁺, but not CD8⁺, T cells are required for efficient antibody-mediated clearance of *B. pertussis*. Together with the previous reports that describe involvement of Th1 response in *B. pertussis* clearance, we propose a model in which CD4⁺, perhaps Th1, T cells facilitate antibody-mediated bacterial clearance by modulating the inflammatory response via cytokines or chemokines that facilitate recruitment of FcγR-bearing cells to overcome the effect of pertussis toxin.
MATERIALS AND METHOD

Bacteria and Mice

*B. pertussis* strain 536 is a streptomycin resistant derivative of Tohama I (6). Bacteria were maintained on Bordet-Gengou (BG) agar (Difco) supplemented with 7.5% defibribrinated sheep’s blood (Remel or Hema Resources) and 20 µg/ml streptomycin. Bacteria were grown in Stainer-Scholte (SS) broth with supplements and 20 µg/ml streptomycin to mid-log phase (optical densities of approximately 0.3 at 600 nm) at 37°C on a roller drum for experiments.

C57BL/6, MuMT, γδ−/−, α−/−, and IFNγ−/− mice were obtained from Jackson Laboratories.

Inoculation, and adoptive transfer protocols

Mice were lightly sedated with isoflurane (IsoFlo-Abbott Laboratories), and 5 X 10^5 CFU of bacteria in 50 µl of PBS were inoculated onto the tip of the external nares. Colonization levels were determined by homogenizing the lungs in 1 X PBS and plating aliquots for colony counts. The homogenates and necessary dilutions were plated in 50 µl volumes onto BG agar with streptomycin. Colonies were counted after 3 days incubation at 37°C. For adoptive transfer experiments, intraperitoneal injection of 200µl of convalescent-phase serum obtained on day 28 post-inoculation from mice which were inoculated with BP 536, into naïve mice was immediately followed by inoculation as described above. Animals were sacrificed on the indicated day post transfer and inoculation and colonization determined as described. Animals were handled in accordance with institutional guidelines. Statistical significance of data points was determined using a student's unpaired t-test.
RESULTS

Adaptive immunity is required for adoptively transferred serum antibody-mediated clearance of *B. pertussis* from the respiratory tract.

Since adoptively transferred serum antibodies were able to clear *B. pertussis* from the respiratory tract of mice by 14 days but not by 7 days post-inoculation, we predicted adaptive immunity developed by 14 days may be facilitating the serum antibody-mediated clearance of *B. pertussis*. In order to test whether or not serum antibody-mediated clearance of *B. pertussis* requires adaptive immunity, we used RAG2−/− mice (17). These mice are defective in T and B cell development due to a null mutation in the recombinase-activating gene 2, which is required for recombination involved in generating B and T cell diversity. This mutation makes the animals defective in the development of adaptive immunity mediated by B and T cells. RAG2−/− mice were adoptively transferred 200-µl of *B. pertussis*-induced convalescent-phase serum or naive serum and inoculated with $5 \times 10^5$ CFU of *B. pertussis* in 50µl of PBS by intra-nasal route. These mice were sacrificed 14 days post-inoculation and the bacterial numbers in the lungs were enumerated. The recovered bacterial numbers were compared with that of similarly treated wild type mice (Fig. 1). The numbers of recovered bacteria from RAG2−/− mice treated with immune serum were 1000-fold greater than that of wild type mice treated with immune serum, indicating that adaptive immunity is required for antibody-mediated clearance of *B. pertussis*.

**T cells but not B cells are required for adoptively transferred serum antibody-mediated clearance of *B. pertussis*.**
Since RAG2−/− mice lack both B and T cells, we sought to determine whether or not B cells or T cells are required for adoptively transferred serum antibody-mediated clearance of B. pertussis. MuMT mice are defective in B cell development due to deletion of exon 1 in the Mu heavy chain (11). We used these mice to determine the role of B cells in adoptively transferred serum antibody-mediated clearance of B. pertussis. A group of MuMT mice were adoptively transferred 200-µl of B. pertussis-induced convalescent-phase serum or naive serum and inoculated with B. pertussis as described previously. The number of bacteria recovered on day 14 post-inoculation was compared with that of similarly treated wild type and RAG2−/− mice (Fig.2). Bacterial numbers recovered from the MuMT mice treated with serum antibodies were equal to the numbers recovered from wild type mice, indicating B cells are not required for adoptively transferred serum antibody-mediated clearance of B. pertussis. Since RAG2−/− but not MuMT mice are defective in the antibody-mediated clearance of B. pertussis, the immune functions that are missing in RAG2−/− but present in MuMT mice appear to enable antibody-mediated clearance of B. pertussis, suggesting a critical role for T cells.

In order to determine the role of T cells in antibody-mediated clearance of B. pertussis, TCRαδ−/− mice, which have defective development of αβ and γδ T cell receptor and cannot form mature αβ and γδ T cells (17), were inoculated with B. pertussis and adoptively transferred 200µl of naïve or convalescent-phase serum as described previously. On day 14 post-inoculation, lungs were harvested and bacterial numbers in the lungs were enumerated. Unlike wild type mice, TCRαδ−/− mice were not able to clear B. pertussis from the lungs even upon adoptive transfer of immune serum (Fig. 2).
indicating αβ and/or γδ bearing T cells are required for antibody-mediated clearance of
*B. pertussis*.

**Adoptively transferred serum antibodies rapidly clear B. pertussis from the respiratory tract of MuMT mice if transferred 14 days post-inoculation.**

Since T cells are required for adoptively transferred serum antibody-mediated clearance of *B. pertussis*, we sought to determine if the already generated T cell response to *B. pertussis* in MuMT mice facilitates rapid clearance of *B. pertussis* by transferred serum antibodies. A group of MuMT mice were inoculated with 5 X10⁵ CFU of *B. pertussis* by intranasal route as described above. On day 14 post-inoculation, 200-µl of convalescent-phase serum was injected i.p. into these mice. On this day MuMT mice harbor approximately 10⁷ bacteria in their lungs (10). On days 17 and 21 post-inoculation, mice were sacrificed and the lungs were harvested for enumeration of *B. pertussis* CFU. On day 17 post-inoculation (day 3 post-adoptive transfer), the number of CFU recovered was 1/100th of the number of CFU recovered from MuMT mice that were treated with naive serum (Fig. 3). By day 21 post-inoculation (day 7 post adoptive-transfer), no bacteria could be recovered from the trachea and lungs of mice that were treated with convalescent-phase serum (Fig. 3). Particularly note, if the immune serum is given at the time of infection as in previous studies, it has no significant effect on bacterial numbers on days 3 and 7 post- treatment. These data suggest that a B- cell independent immune response, generated in MuMT mice by 14 days post-inoculation, facilitates rapid antibody-mediated clearance of *B. pertussis*, and implicates T cells in this process.
αβ T cells are required for adoptively transferred serum antibody-mediated clearance of *B. pertussis*

In order to determine the importance of αβ T cells in serum antibody-mediated bacterial clearance, TCRα−/− and congenic C57BL/6 mice were inoculated with *B. pertussis* as described previously and adoptively transferred 200-µl of naïve serum or immune serum. On day 14 post-inoculation, these mice were sacrificed and bacteria in the lungs were enumerated. While immune serum completely cleared *B. pertussis* in the lungs of wild type mice, it had no effect on bacterial numbers in TCRα−/− mice (Fig. 4) suggesting that αβ-bearng T cells are required for adoptively transferred serum antibody-mediated clearance of *B. pertussis*.

**CD4⁺, but not CD8⁺, T cells are required for adoptively transferred serum antibody-mediated clearance of *B. pertussis***

We sought to determine the subset of αβ T cells that may facilitate antibody-mediated clearance of *B. pertussis* using anti-CD4 and anti-CD8 monoclonal antibodies. Previous reports have indicated that 1 mg of these antibodies depletes either CD4⁺ or CD8⁺ T cells for at least 14 days post-treatment in a mouse model. Groups of wild-type and MuMT mice were inoculated with *B. pertussis* as described previously and adoptively transferred 200–µl of naïve or immune serum. Subsequently, 1 mg of PBS, anti-CD4 or ant-CD8 mAbs was injected intraperitoneally. On day 14 post-inoculation, these mice were sacrificed and bacterial numbers in the lungs were measured. Serum antibodies cleared *B. pertussis* in the lungs of PBS treated wild type and MuMT mice as well as anti-CD8 mAb treated wild type and MuMT mice (Fig. 5). However, serum antibodies failed to eliminate bacteria in the lungs of wild type and MuMT mice that
were treated with anti-CD4 mAb suggesting that CD4<sup>+</sup> T cells are required for antibody-mediated clearance of *B. pertussis*.

**IFNγ is necessary for adoptively transferred antibody-mediated clearance of *B. pertussis***

Previous reports have shown that CD4<sup>+</sup>, but not CD8<sup>+</sup>, T cells and Th1 type response are necessary for *B. pertussis* clearance (1 & 2). Furthermore, Barbic et al (2) have shown that IFNγ, a Th1 cytokine, is necessary for natural clearance of *B. pertussis*. Therefore, we hypothesized that Th1 cytokines such as IFNγ and IL-12 produced by Th1 cells facilitate antibody-mediated clearance of *B. pertussis*. Groups of IFNγ<sup>-/-</sup> and wild type C57BL/6 mice were inoculated with *B. pertussis* and adoptively transferred 200µl of either naïve or immune serum. On day 14 post-inoculation, mice were sacrificed and the numbers of bacteria in the lungs were measured. Immune serum cleared bacteria in the lungs of wild type but not IFNγ<sup>-/-</sup> mice indicating IFNγ is necessary for antibody-mediated clearance of *B. pertussis* (Fig. 6). However, since a defective T cell response is noted in IFNγ<sup>-/-</sup> mice, we will confirm these data by neutralizing IFNγ in wild type and MuMT mice. We are currently investigating the function of IFNγ which may be required for overcoming the effect of pertussis toxin and facilitate antibody-mediated clearance of *B. pertussis*.
DISCUSSION

Epidemiological studies have indicated that *B. pertussis* survives in the immune population (4, 12). The ability of bacteria to persist in a vaccinated or immune population is intriguing, albeit disturbing, as it poses a potential health risk for the unvaccinated, immunocompromised and elderly populations. This concern is substantiated by the fact that *B. pertussis* is reemerging among children, and surprisingly, among the adolescent population (21). Furthermore, a recurring epidemic pattern of whooping cough is seen every 3 to 4 years in vaccinated populations (20). One possible explanation is that current vaccines cannot induce longstanding immunity, and waning immunity allows for reinfection by the pathogen (5). Additionally, there is growing evidence that *B. pertussis* can transiently infect immune individuals and can be sustained in the population without causing disease until a susceptible individual is encountered (20). One potential reason for this is that although current Pa induce a strong antibody response, antibodies have limited effect on the bacterial colonization during the initial infection period (10).

Recently, we have demonstrated that expression of pertussis toxin allows *B. pertussis* to transiently resist antibody-mediated bacterial clearance in the respiratory tract (Chapter 5).

B cells have been shown to be necessary for infection-induced as well as vaccine-induced immunity in a mouse model (13), suggesting a critical role for antibodies in immunity to *B. pertussis*. However, the role of antibodies in immunity to *B. pertussis* remains controversial since large human clinical studies have failed to find a correlation between antibody titer and protection (19). These findings coupled with the failure of serum antibodies to clear *B. pertussis* from the respiratory tract of mice up to one week
post-inoculation prompted several groups to propose that B cells are required for either antigen presentation or cytokine secretion to aid in T cell dependent immune responses to *B. pertussis* (13). However, previous research on immunity to *B. pertussis* focused on T cells without a detailed understanding of the mechanism by which T cells might clear an extracellular mucosal pathogen. Our data show that antibodies are required for clearance, supporting a model in which CD4^+^ T cells are required for efficient antibody-mediated clearance of *B. pertussis*.

Barbic *et al* (2) have previously shown that IFNγ^-/-^ mice as well as IFNγ Receptor^-/-^ mice are unable to clear *B. pertussis* from the respiratory tract. Mills *et al* (16) also confirmed these findings in an independent study. These studies indicate that IFNγ is necessary for *B. pertussis* clearance, but do not address the mechanism(s) involved. Similarly, IL-12p40^-/-^ mice show delayed clearance of *B. pertussis* (16), suggesting IL-12 is also important for immunity to *B. pertussis*. The authors of these previous works concluded that T cells have complex interactions that include T helper, T regulatory, and T cytotoxic cells. However, since *B. pertussis* is an extracellular bacterium, found within the lumen of airways, there has been a substantial discrepancy between the observed generation of these T cell subsets, and any clear explanation of mechanisms by which they may be eliminating bacteria from airways. Based on our data that reveal a role for T cells in antibody-mediated bacterial clearance, we propose that T cells produce IFNγ, and IL-12 that triggers recruitment and activation of FcγR bearing cells, increases phagocytosis of antibody bound *B. pertussis*, and facilitates intracellular bactericidal activity.
Inclusion of pertussis toxin in the pertussis vaccine is critical, suggesting the importance of pertussis toxin specific antibodies in protection against whooping cough. As further support, a recent report that studied a large vaccinated population in Europe has shown that protection correlates with anti-pertussis toxin antibody titers (7). Our previous studies suggest the importance of antibody response that neutralizes pertussis toxin for sterilizing immunity (Chapter 5). In contrast, T cells, particularly CD4⁺ T cells, have been shown to be necessary to control and clear *B. pertussis* from the respiratory tract of mice (13). Similarly, in human studies a robust CD4⁺ T cell response has been noted in association with the protective immunity observed after infection or vaccination with Pw (3). In this report we show that CD4⁺ T cell-dependent immunity can overcome the effect of pertussis toxin and eliminate *B. pertussis* from the respiratory tract. Together, these data support vaccination strategies that induce long lasting pertussis toxin-specific antibodies and a robust Th1 response in order to provide herd immunity and prevent epidemics.
Fig. 1 T and B cells are required for adoptively transferred serum antibody-mediated clearance of *B. pertussis*. Wild type and RAG2−/− mice were inoculated with *B. pertussis* and adoptively transferred naïve or immune serum. Lungs were harvested and bacteria enumerated on day 14 post-inoculation.
Fig. 2 T cells are required for serum antibody-mediated clearance of *B. pertussis*.

Wild type, MuMT and TcRαδ^−/−^ mice were inoculated with *B. pertussis* and adoptively transferred naïve or immune serum. Lungs were harvested on day 14 post-inoculation to enumerate bacteria.
Fig. 3 T cell response is sufficient to facilitate adoptively transferred serum antibody-mediated clearance of *B. pertussis*. MuMT mice were inoculated with *B. pertussis*. On day 14 post-inoculation, these mice were adoptively transferred 200µl of either naïve or immune serum. On days 17 (3 days post-treatment) and 21 (7 days post-treatment), lungs were collected to enumerate bacteria.
Fig. 4 αβ T cells are required for serum antibody-mediated clearance of *B. pertussis*.

Wild type and TcRα−/− mice were inoculated with *B. pertussis* and adoptively transferred naïve or immune serum. Lungs were harvested on day 14 post-inoculation to enumerate bacteria.
Fig. 5 CD4+ T cells are required for antibody-mediated clearance of B. pertussis.

Groups of MuMT mice were inoculated with *B. pertussis* and adoptive transferred with 200µl of naïve or immune serum. Subsequently, 1 mg of anti-CD4 or anti-CD8 antibodies was injected and bacterial numbers in the lungs were measured on day 14 post-inoculation.
Fig. 6 IFNγ is required for adoptively transferred serum antibody-mediated clearance of *B. pertussis*. Wild type and IFNγ<sup>-/-</sup> mice were inoculated with *B. pertussis* and adoptively transferred naïve or immune serum. Lungs were harvested on day 14 post-inoculation to enumerate bacteria.
REFERENCES


caused by Bordetella parapertussis compared with illness caused by Bordetella pertussis.


CHAPTER 7

DISCUSSION:

IMMUNE RESPONSE TO *Bordetella* Spp.
INTRODUCTION

The classical members of the genus *Bordetella* include *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (34). It is believed that human adapted *B. pertussis* and *B. parapertussis* have emerged from a *B. bronchiseptica*-like progenitor in two separate events (30, 41). All three subspecies share a similar set of virulence factors yet differ in host adaptation and pathogenesis (31). *B. pertussis* causes whooping cough, primarily in young children of 1-10 years of age and is responsible for the death of approximately 300,000 children every year (6, 42), mostly in developing nations. *B. parapertussis* causes acute respiratory disease similar to whooping cough and hence the incidence of *B. parapertussis* infection is often underestimated (16, 44). *B. bronchiseptica* can chronically infect a variety of non-human mammals without any overt symptoms but also causes respiratory diseases in pigs (atrophic rhinitis), dogs (kennel cough), and rabbits (snuffles) (9, 34). It has also been isolated from immunocompromised humans including HIV patients among whom 0.3% of respiratory complications have been associated with *B. bronchiseptica* (40).

The current vaccines to *B. pertussis* and *B. bronchiseptica* are effective in protecting from disease but not from infection (6). Consequently, these bordetellae have been isolated even among vaccinated human/animal populations suggesting bacteria can persist in a population by subclinically infecting immune host (8, 38). Furthermore, pertussis vaccines provide little protection against *B. parapertussis* against which no vaccines are available (43). Hence, in order to prevent epidemics, it is essential to design novel vaccine strategies that provide sterilizing and cross immunity to *Bordetella* spp. It is evident from our work as well as the work of other groups that antibodies play a major
role in clearance of bordetellae in the respiratory tract (13, 14, and 20). Therefore, it is critical to understand the mechanism of antibody-mediated bacterial clearance in various respiratory organs that may aid in the rational designing of therapeutics and vaccines.

The *B. bronchiseptica*-mouse model has been extensively used to study the interactions of a natural pathogen with mucosal immune responses (9). Significant advantages of this model include the availability of a range of specific virulence factor deficient strains derived from a common clinical isolate of *B. bronchiseptica* and the availability of an array of mice with defined immune deficiencies. Thus, the contribution of specific virulence factor and immune function to the outcome of a host-pathogen interaction can be easily investigated. Although as few as 5 CFU of *B. bronchiseptica* can infect mice (9), use of high dose-high volume inoculation protocol used in this study allows for simultaneous investigation of interactions in various respiratory organs with consistent outcomes that can be easily measured. Since this model represents a natural host-pathogen interaction, the knowledge gained may be applicable to other relevant respiratory pathogens.

*B. pertussis* and *B. parapertussis* also efficiently colonize the respiratory tract of mice (11, 26, and 43) and as such the murine model has been extensively used to study the biology of these bordetellae. Comparative immunobiology of these three closely related subspecies of *Bordetella* in a common murine model provides an excellent opportunity to study molecular mechanism of the infection process and disease progression. In this study, we have determined the mechanism of rapid antibody-mediated clearance of *B. bronchiseptica* in great detail. Using the comparative approach, we have shown that human adapted bordetellae modulate inflammatory responses and
thereby resists rapid antibody-mediated bacterial clearance. However, the mechanism of resistance to rapid clearance appears to be distinct between two independently emerged bordetellae suggestive of the convergent evolution of human adapted *Bordetella*. 
IMMUNE RESPONSE TO *B. BRONCHISEPTICA*

Innate immune response.

In vitro studies have demonstrated that *B. bronchiseptica* LPS induces a robust TLR4-mediated inflammatory response characterized by high level of pro-inflammatory cytokines and chemokines such as TNFα, IL-1β, IL-6, KC, MIP1α, MCP, and etc (22). Similarly, in vivo studies have indicated that *B. bronchiseptica* induces a robust TLR4-mediated inflammatory response and an early TLR4-dependent TNFα response is necessary for controlling and limiting bacterial numbers in the respiratory tract (21). The function of TNFα in controlling bacteria in the respiratory tract remains to be studied.

We have investigated the importance of inflammatory cells such as alveolar macrophages and neutrophils in controlling *B. bronchiseptica* in the respiratory tract. While macrophages appear to be critical for the production of various pro-inflammatory cytokines and chemokines, an early TLR4-dependent neutrophil response is necessary for controlling bacterial numbers (Chapter 4 and Mann PB and Harvill ET, Unpublished data). Together, these data suggest that *B. bronchiseptica* induces a TLR4-mediated inflammatory response, primarily by macrophages, resulting in the induction of cytokines such as TNFα and chemokines such as KC. Subsequently, a robust PMN response controls bacterial numbers and thus limits damage by cytotoxic bacterial virulence factors.

Adaptive immune response.

*B. bronchiseptica* is lethal in RAG2<sup>−/−</sup> and SCID mice that lack both B and T cells (9). These mice fail to reduce bacterial numbers and restrict bacteria in the respiratory tract (9). B cell deficient mice are able to limit bacteria to the respiratory tract and hence
do not succumb to infection (13). But B cell deficient mice fail to reduce bacterial numbers suggesting that antibodies may be necessary for bacterial clearance (13). Concomitantly, our data indicate that antibodies are critical in clearing bacteria in the respiratory tract since adoptive transfer of serum antibodies into B cell deficient mice reduces bacterial numbers in the lower respiratory tract (13) and IgA deficient mice fail to efficiently reduce bacterial numbers in the upper respiratory tract (Chapter 3). Together these data suggest that serum antibodies are necessary and sufficient to clear *B. bronchiseptica* in the lungs and trachea, whereas secretory IgA is required to reduce the number of bacteria in the upper respiratory tract.

T cell deficient, similar to B cell deficient, mice also fail to reduce bacterial numbers but are able to restrict bacteria to the respiratory tract (Pilione M and Harvill ET, Unpublished data). The exact function of T cells in bacterial clearance is yet to be determined but a poor antibody response to *B. bronchiseptica* has been observed in T cell deficient mice (Pilione M and Harvill ET, Unpublished data). Adoptive transfer of serum antibodies from T cell deficient mice fail to clear *B. bronchiseptica* from the lungs and trachea of naïve wild type mice (Pilione M and Harvill ET, Unpublished data). However, adoptive transfer of convalescent-wild type mouse serum into T cell deficient mice reduces bacterial numbers in the lower respiratory tract suggesting that T cells are not required for antibody functions but may be required for generation of antibodies (Kirimianjeswara GS and Harvill ET, Unpublished data). It is also important to note that T cell-deficient mice may also have other immune deficiencies that may affect host immunity to *B. bronchiseptica*. It is interesting to note *B. bronchiseptica* clearance is delayed in IFNγ−/− mice, perhaps due to a defective T cell response and hence defective
antibody generation (Pilione M and Harvill ET, Unpublished data). These data suggest that adaptive immunity, particularly antibodies, are critical in reducing bacterial numbers in the respiratory tract.

**Antibody-mediated clearance of *B. bronchiseptica***

In light of the importance of antibodies for *B. bronchiseptica* clearance in the respiratory tract, determination of the mechanism by which antibodies clear bacteria is essential to design vaccines that provide sterilizing immunity. Passive transfer of convalescent-phase serum into naïve mice allows us to elucidate the mechanism of antibody-mediated bacterial clearance in the absence of other adaptive immune functions. Serum antibodies rapidly clear *B. bronchiseptica* in the trachea and lungs of wild type, RAG2−/−, MuMT, and TCRα−/− mice suggesting that innate immune responses are sufficient to facilitate antibody-mediated bacterial clearance (Kirimanjeswara GS and Harvill ET, Unpublished data). The innate immune responses that are necessary for antibody-mediated bacterial clearance include a TLR4-mediated early PMN response (Chapter 4). Interestingly, a TLR4-dependent TNFα response was not necessary for antibody-mediated bacterial clearance although it was necessary for controlling bacterial numbers in naïve mice (Kirimanjeswara GS and Harvill ET, Unpublished data and 21). It is worth noting that TNFα was not required for neutrophils recruitment (Mann PB and Harvill ET, Unpublished data). Further investigation of the mechanism of antibody-mediated bacterial clearance revealed that complement component C3, but not C5, is required, suggesting that MAC-dependent lysis had minimal effect on bacterial clearance (33 and Chapter 4). Both C3−/− and C5−/− mice appeared to have normal inflammatory response to *B. bronchiseptica* suggesting anaphylotoxins C3a and C5a are not required.
for antibody-mediated bacterial clearance (33). However, CR3 and FcγRs were found to be necessary for antibody-mediated bacterial clearance (33 and Chapter 4). Taken together, these data suggest that *B. bronchiseptica* induces TLR4-dependent recruitment of neutrophil to the lungs which phagocytose antibody bound bacteria via FcγRs and CR3. Deficiency in either 5-Lipoxygenase (5-LOX) or 15-Lipoxygenase (15-LOX) did not affect antibody-mediated bacterial clearance suggesting PMN recruitment was independent of LTB4, LTB5, C3a, and C5a (Kirimanjeswara GS and Harvill ET, Unpublished data). However, it is worth mentioning that compensatory and redundant mechanisms of neutrophil recruitment in the absence of each of these chemoattractants may be sufficient for bacterial clearance. Interestingly, complement was not required for antibody-mediated clearance of an O-ag mutant of *B. bronchiseptica* suggesting that the O-ag either prevents phagocytosis of opsonized bacteria or access to other antibodies and we are currently investigating this phenomenon (Kirimanjeswara GS and Harvill ET, Unpublished data).

A similar mechanism of antibody-mediated bacterial clearance was involved in the trachea except that FcγRs were not necessary (Chapter 4). Furthermore, serum IgA appears to be required for antibody-mediated bacterial clearance in the trachea (Chapter 3), whereas IgG isotypes are involved in the lungs Pilione M and Harvill ET, Unpublished data). However, how IgA mediates bacterial clearance is not known. Since C3, CR3, and neutrophils are also required in the trachea, we hypothesize that IgA activates complement cascade and bacteria are phagocytosed via CR3 by neutrophils. In support of this hypothesis, IgA has recently been shown to activate the classical pathway of complement (36). These organ specific immune functions that facilitate pathogen
clearance have been previously observed in *Pseudomonas* pathogenesis where complement was critical in the trachea and FcγRs were critical in the lungs (1).

Adoptively transferred serum antibodies have little effect on the numbers of *B. bronchiseptica* in the upper respiratory tract (13). On the other hand, the reduction in bacterial numbers in the nasal cavity is facilitated by mucosal IgA (Chapter 3). It is worth noting bacterial numbers are reduced, albeit slowly, in the absence of IgA suggesting that other mucosal antibodies can also facilitate bacterial clearance (Chapter 3). Thus serum antibodies appear to be effective in organs where immune surveillance is maximum while IgA appears to be important in immune privileged sites.
IMMUNE RESPONSE TO *B. PERTUSSIS*

Innate immune response

*B. pertussis* has been shown to induce a TLR4-mediated inflammatory response but unlike *B. bronchiseptica*, is not lethal to TLR4 deficient mice (12). These mice exhibit a transient defect in bacterial clearance and a defective IL-10 response, leading the authors to suggest that TLR4 mediated response is required for down-regulating *B. pertussis* induced pathology that supports the growth of bacteria (12). TLR4 deficient mice also had a defective TNFα and IFNγ response (12). We have shown that the TNFα response is necessary to control and limit *B. pertussis* in the respiratory tract but a TLR4-independent TNFα response appears to be sufficient to control bacterial numbers (Mann PB and Harvill ET, Unpublished data). Similar to TNFα⁻/⁻ mice, IFNγR⁻/⁻ mice succumb to *B. pertussis* infection leading the authors to suggest that IFNγ is required for preventing dissemination of bacteria to the systemic sites (17). In support of these data, a recent report indicates that depletion of NK cells results in a marked reduction of IFNγ in the lungs that leads to disseminated disease by *B. pertussis* (3). However, IFNγ⁻/⁻ mice do not succumb to *B. pertussis* but only fail to clear bacteria in the lungs (17). Similarly, IL-12p40⁻/⁻ mice, which survive *B. pertussis* infection, produce significantly less IFNγ than wild type mice, but only have a protracted kinetics of bacterial clearance (19). It is also interesting to note that NK cell recruitment peaks only after day 14 post-inoculation but IFNγR⁻/⁻ mice die much earlier (3). Thus the role of IFNγ remains controversial. The conflicting reports about the role of IFNγ may partly be due to different strains of mice, strains of *B. pertussis* and inoculation protocols used by various investigators. It is yet to
be determined if the recruitment of IFNγ secreting cells including NK cells, and secretion of IFNγ by these cells, are affected by TNFα.

Innate immune cells such as alveolar macrophages do not appear to be critical in protecting mice from lethal *B. pertussis* infection (Mann PB and Harvill ET, Unpublished data). The contribution of resident macrophages to the cytokine and chemokine responses is yet to be determined. Neutrophils, however, may play a major role in controlling *B. pertussis* numbers early during infection (4). This is consistent with our findings that *B. pertussis* is able to inhibit the recruitment of neutrophils to the lungs by expressing pertussis toxin (39). Furthermore, depletion of neutrophils allows for 100 fold increase in the number of *B. pertussis* Δptx suggesting that pertussis toxin facilitates initial *B. pertussis* colonization (Chapter 5). However, neutrophil numbers begin to peak between day 7 and 14 post-inoculation and we propose that a Th1 response may overcome the effect of pertussis toxin and allows for the recruitment and/or activation of neutrophils.

**Adaptive immune response**

The adaptive immune response to *B. pertussis* has been investigated to greater detail (26). Similar to *B. bronchiseptica*, *B. pertussis* clearance requires both B and T cells and in the absence of adaptive immunity (14, 27), mice succumb to the infection. T cell deficient mice fail to clear *B. pertussis* in the lungs and thus have a protracted disease (27). Adoptive transfer of CD4+ T cells into T cell deficient mice reduced *B. pertussis* numbers in the lungs indicating a critical role for CD4+ T cells in protective immunity (14, 35). *B. pertussis* induces a Th1 type immune response in mice, consistent with similar clinical findings in humans (37). A Th1 response is also associated with protective immunity to *B. pertussis*, either by prior infection or vaccination with Pw (37).
However, an exact role for these cells has not been established. A recent report also suggests that T regulatory cells play a major role in controlling *B. pertussis* induced pathology and hence are involved in protective immunity to *B. pertussis* (24).

We and others have shown that B cell deficient mice fail to clear *B. pertussis* in the respiratory tract (13, 14, and 20). Since adoptive transfer of serum antibodies failed to clear *B. pertussis* in these mice for up to two weeks post-inoculation, Shahin et al suggested that B cells are required for functions other than producing antibodies (14). However, Mills et al have shown that B cells as well as CD4$^+$ T cells are required for bacterial clearance in the lungs and concluded that *B. pertussis* may exist as both intra- and extra-cellular pathogen and T cell response may eliminate the former while antibodies clear the latter form of bacteria (35). However, the existence of intracellular bacteria has not been proven experimentally but is mainly based on circumstantial evidence. Furthermore, depletion of CD8$^+$ T cells, a major cell population that is associated with the killing of intracellular bacteria, has only a marginal effect on *B. pertussis* clearance (20). Various reports also have indicated that physiologically relevant cells such as alveolar macrophages and neutrophils poorly support the intracellular survival of *B. pertussis* (15, 23). Additionally, Pa, which mainly induces serum antibodies, provides protection from the disease (25). In this study we have shown that mucosal IgA is not necessary and serum antibodies efficiently reduce bacteria in the respiratory tract, albeit slowly, emphasizing the importance of serum antibodies in protective immunity to *B. pertussis* (Chapter 3). We have additionally shown that a T cell response, perhaps a Th1 type, is required for effective antibody-mediate clearance of *B. pertussis* (Chapter 6). Therefore, we propose a defined model to explain the requirement
of both B and T cells in the bacterial clearance: B cells are required for generation of antibodies, which facilitate FcγR-mediated phagocytosis of bacteria by neutrophils (see below), and T cells are required for the recruitment and/or activation of neutrophils.

**Antibody-mediated clearance of B. pertussis**

Adoptively transferred serum antibodies fail to rapidly (<3 days) clear *B. pertussis* from the lungs and trachea and have no effect on bacterial numbers during the first 7 days post-inoculation (13). However, serum antibodies significantly reduce the number of bacteria in the lungs on day 10 and completely clear bacteria on day 14 post-inoculation (Chapter 5). Thus, we investigated the mechanism of antibody-mediated clearance of *B. pertussis* on day 14 post-inoculation. Serum antibodies clear *B. pertussis* in wild type and MuMT mice but not in RAG2−/− and TCRα−/− mice (Chapter 6), suggesting that innate immune responses are not sufficient to facilitate antibody-mediated *B. pertussis* clearance. Adoptive transfer of convalescent-phase serum together with T cells from convalescent- MuMT mice facilitated *B. pertussis* clearance in RAG2−/− or TCRα−/− mice (Chapter 6), indicating that T cells are required for antibody-mediated bacterial clearance. Interestingly, serum antibodies failed to clear *B. pertussis* in IFNγ−/− mice (Chapter 6). Together, these data suggest that cytokine/s such as IFNγ produced by T cells facilitate antibody-mediated clearance of *B. pertussis* by yet an unknown mechanism.

Antibody-mediated clearance of *B. pertussis* requires FcγRs, but not C3 or C5 (Chapter 5). Consistent with these findings, depletion of neutrophils abrogated the effects of serum antibodies on bacterial numbers suggesting that opsonized *B. pertussis* is phagocytosed via FcγRs on neutrophils (Chapter 6). Interestingly, the recruitment of neutrophils peaks only after day 7 post-inoculation, a timeline correlating with antibody-
mediated bacterial clearance, raising the possibility that *B. pertussis* may inhibit neutrophil recruitment to the lungs. In fact, *B. pertussis* has been shown to induce a significantly less inflammation than *B. bronchiseptica* early during infection (9). Therefore, we predicted that a *B. pertussis* specific virulence factor, pertussis toxin, may be responsible for inhibition of neutrophil recruitment. Indeed, *B. pertussis*Δptx induced a rapid neutrophil response in the lungs and consequently antibodies rapidly cleared this mutant bacterium (chapter 5). In vitro data also indicated that pertussis toxin inhibited neutrophil migration (chapter 5). Since, pertussis toxin is an inhibitor of G protein coupled receptor signaling (28, 29), it may be affecting neutrophil migration by inhibiting chemokine receptors and/or affecting adhesion molecule expression on endothelial cells and neutrophils. However, as mentioned before, a T cell response may overcome the effect of pertussis toxin and induce a neutrophil response facilitating antibody-mediated *B. pertussis* clearance. Consistent with this model, adoptively transferred serum antibodies fail to clear *B. pertussis* in TLR4-deficient mice (Kirimianjeswara GS and Harvill ET, Unpublished data), which have been shown to be defective in production of various cytokine including IFNγ, and may also have a defective Th1 response.

Serum antibodies have little effect on *B. pertussis* numbers in the upper respiratory tract (13). However, IgA−/−, but not MuMT, mice clear bacteria from the nasal cavity (Chapter 3). These data suggest that non-IgA antibodies such as IgG2b and IgM produced at the mucosal surface are sufficient to clear *B. pertussis* from the nasal cavity. Therefore we propose that the induction of serum antibodies and a Th1 response may be sufficient to provide sterilizing immunity to *B. pertussis* in the lungs and trachea, the
natural sites of colonization of the bacterium, while mucosal antibodies are required for bacterial clearance in the nasal cavity.
Innate immune response

*B. parapertussis* induces a TLR4-dependent inflammatory response, although significantly less than that of *B. bronchiseptica* (Mann PB and Harvill ET, Unpublished data). However, TLR4-deficient mice do not succumb to *B. parapertussis* and no apparent defect is observed in bacterial clearance (Mann PB and Harvill ET, Unpublished data). Similar to wild type mice, TNFα\(^{-/-}\) mice are able to control and reduce *B. parapertussis* suggesting that TNFα may not play a major role in immunity to *B. parapertussis* (Mann PB and Harvill ET, Unpublished data). However, whether TNFα is involved in the induction of protective immunity has not been determined. *B. parapertussis* induces a very low level of neutrophil recruitment to the lungs (Wolfe D and Harvill ET, Unpublished data) and depletion of neutrophils has no major effect on bacterial numbers (Wolfe D and Harvill ET, Unpublished data). Depletion of macrophages results in increased pathology but does not affect bacterial numbers (Wolfe...
D and Harvill ET, Unpublished data). The innate immune response to *B. parapertussis* thus appears to be different from that mounted against other two bordetellae.

**Adaptive immune response**

Similar to *B. bronchiseptica*, *B. parapertussis* causes lethal infection in RAG2<sup>−/−</sup> mice (9 and Wolfe D and Harvill ET, Unpublished data). Furthermore, both B and T cells are required for *B. parapertussis* clearance in the respiratory tract (13 and Wolfe D and Harvill ET, Unpublished data). IgA<sup>−/−</sup>, but not MuMT, mice were near normal in their ability to clear *B. parapertussis* (Chapter 3) and adoptively transferred serum antibodies were effective in clearing this bacterium from the lower respiratory tract after the generation of a T cell response (Kirimaneswara GS, Wolfe D and Harvill ET, Unpublished data). These data suggest that immunity to *B. parapertussis* primarily depends on serum antibodies and serum antibodies require a T cell response for bacterial clearance in the respiratory tract.

**Antibody-mediated clearance of *B. parapertussis***

The incidence of *B. parapertussis* infections is on the rise in populations previously vaccinated with Pa (44). Thus, it appears that serum antibodies generated against *B. pertussis* have little effect on *B. parapertussis*. Our recent studies indicate that antibodies raised against *B. pertussis* have little cross reactivity to *B. parapertussis* (Wolfe D and Harvill ET, Unpublished data). Furthermore, our data indicate that there is little cross protection between these species (Wolfe D and Harvill ET, Unpublished data). Therefore it is essential to understand the mechanism of protective immune responses such as serum antibodies, which are required for *B. parapertussis* clearance, to design vaccines that can provide protection from infection by both bordetellae.
The mechanism of serum antibody-mediated clearance of *B. parapertussis* closely resembles that of *B. pertussis*. Serum antibodies begin reducing the number of *B. parapertussis* only after day 7 post-inoculation (Kirimanjeswara GS, Wolfe D and Harvill ET, Unpublished data). The complete clearance of *B. parapertussis* requires T cells, IFNγ, FcγRs, and neutrophils (Wolfe D and Harvill ET, Unpublished data). Additionally, antibody-mediated clearance of *B. parapertussis*, similar to *B. bronchiseptica*, requires C3 and CR3 (Wolfe D and Harvill ET, Unpublished data). Together these data suggest that antibodies clear *B. parapertussis* via CR3 and FcγR-mediated phagocytosis by neutrophils. However, the neutrophil response to *B. parapertussis* is significantly delayed compared to that of *B. bronchiseptica* and hence antibodies are effective only after day 7 post-inoculation (Wolfe D and Harvill ET, Unpublished data). Since *B. parapertussis* does not express pertussis toxin, one possible reason for this delayed and subdued neutrophil recruitment could be that *B. parapertussis* induces significantly less inflammation than the other two bordetellae. Later during infection the T cell, perhaps Th1, response recruits and activates neutrophils and thus facilitates antibody-mediated clearance of bacteria.
COMPARITIVE IMMUNOBIOLOGY OF BORDETELLAE

The innate immune responses, required to control bacterial numbers, significantly differ for *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis*. This in part may be due to modification of their LPS structures. For instance, as little as 0.1 ng of *B. bronchiseptica* LPS is sufficient to elicit a detectable pro-inflammatory response by macrophages but approximately 10ng of *B. pertussis* LPS and 100 ng of *B. parapertussis* LPS is required to elicit a comparable response (Mann PB and Harvill ET, Unpublished data paul). The differential reactivity of LPS may be associated with acylation status and other yet unknown structural differences of their LPS. Consistent with these in vitro findings, *B. bronchiseptica* induces a highest degree of inflammation in vivo, as determined by TNFα level and histopathology, followed by *B. pertussis* and *B. parapertussis* (Mann PB and Harvill ET, Unpublished data). Accordingly, TLR4 was necessary to control *B. bronchiseptica* numbers in the respiratory tract and protect mice from lethality (22). TLR4-deficient mice survived *B. pertussis* infection and showed only a transient defect in controlling bacterial numbers (12). TLR4 was neither required to control *B. parapertussis* numbers nor to protect mice from lethal infection (Mann PB and Harvill ET, Unpublished data). This interesting contrast was also observed in the requirement for TNFα in controlling bacterial numbers. TLR4-dependent early TNFα was necessary to control the numbers of *B. bronchiseptica*, but not *B. pertussis* or *B. parapertussis*. However, TNFα was required for controlling *B. pertussis*, but not *B. parapertussis*, numbers later during infection (paul and dan).

*B. bronchiseptica* and *B. parapertussis* LPS possess O-ag structure and are resistant to complement-mediated lysis in vitro (2). In vivo studies have shown that O-ag
is required for *B. bronchiseptica* and *B. parapertussis* to spread to the systemic sites in SCID mice, perhaps via blood (2). *B. pertussis* lacks an O-ag structure and is sensitive to complement in vitro (32). Accordingly, *B. pertussis* has not been isolated from systemic sites or blood of SCID mice (9). Interestingly, *B. bronchiseptica* strains isolated from humans with chronic respiratory disease appear to have lost O-ag and physiological significance of this structural difference is not known (7).

Innate immune cells such as macrophages are critical in controlling *B. bronchiseptica*, perhaps for secretion of inflammatory cytokines and chemokines (Mann PB and Harvill ET, Unpublished data). However, depletion of macrophages had no significant effect on the control of *B. pertussis* or *B. parapertussis* (Wolfe D, Mann PB, and Harvill ET, Unpublished data). An early TLR4-dependent neutrophil response was observed in mice infected with *B. bronchiseptica*, but not *B. pertussis* or *B. parapertussis* (Chapter 4, 5, Wolfe D and Harvill ET, Unpublished data). This neutrophil response was critical in controlling *B. bronchiseptica*, but not *B. pertussis* or *B. parapertussis*, in the lungs (Mann PB, Wolfe D, and Harvill ET, Unpublished data). However, a neutrophil peak is observed around 7-10 days post-inoculation in the lungs of mice infected with *B. pertussis* or *B. parapertussis* but is not required for controlling bacterial numbers (Chapter 5, Wolfe D and Harvill ET, Unpublished data).

Adaptive immune responses are required to reduce bacterial numbers and, in contrast to innate immune responses, appear to be similar for all three bordetellae. For instance, B and T cells are required for natural clearance and also for protective immunity to all three bordetellae (13, 35, and Pilione, M, Wolfe D, and Harvill ET, Unpublished data). They all induce a robust Th1 type response that plays a major role in either
generation and/or function of serum antibodies (13). Serum antibodies are necessary to reduce the number of all three bacteria in the lungs and trachea (13). However, secretory IgA is necessary for reducing the numbers of *B. bronchiseptica*, but not *B. pertussis* and *B. parapertussis*, in the upper respiratory tract (Chapter 3). Similarly, protective immunity to *B. bronchiseptica*, but not *B. pertussis* or *B. parapertussis*, requires IgA in the upper respiratory tract (Chapter 3).

Elucidation of the mechanism of antibody-mediated clearance of bordetellae, using an adoptive transfer approach, further helped in finding clues to the molecular aspects of adaptation and pathogenesis. *B. bronchiseptica* infection results in rapid recruitment of neutrophils which phagocytose antibody bound bacteria via FcγR and CR3 (33, Chapter 4). However, during *B. pertussis* and *B. parapertussis* infections, neutrophil response is slower and appears to be dependent on T cell help (Chapter 5 and Wolfe D and Harvill ET, Unpublished data). Consequently, antibodies clear these pathogens later during infection (>7 days). Interestingly, *B. pertussis* actively inhibits neutrophil recruitment by expressing pertussis toxin (Chapter 5). Additionally, *B. pertussis* LPS induces comparatively less inflammation than that of *B. bronchiseptica*, and hence the delayed neutrophil response may be due to a combination of pertussis toxin and modified LPS (Mann PB and Harvill ET, Unpublished data Paul). However, the limited early neutrophil response during *B. parapertussis* infection may be due to its modified LPS structure alone, since co-inoculation of *B. bronchiseptica* facilitates rapid antibody-mediated clearance of *B. parapertussis* (Wolfe D and Harvill ET, Unpublished data). These data indicate that human adapted bordetellae have acquired independent and overlapping mechanisms of down-regulating early inflammatory responses.
SIGNIFICANCE

*B. pertussis* is highly contagious and hence, the majority of human populations are immune due to either prior infection or vaccination. *B. pertussis* has successfully evolved to survive in immune populations as evidenced by periodic epidemics of whooping cough and high incidence of subclinical infections among immune populations (8). Furthermore, the incidence of pertussis appears to be on the rise among adolescent and elderly populations emphasizing the shift in the characteristics of epidemics by *B. pertussis* (6). In order to eradicate the pathogen, it is necessary to induce a prolonged sterilizing immunity. Accordingly, new vaccination strategies such as mucosal vaccines and vaccination of adults every 10 years have been proposed (5). Our data suggest that *B. pertussis* is able to resist the effects of serum antibodies, primarily by expressing pertussis toxin, enabling it to transiently infect an immune host. These strategies may have allowed *B. pertussis* to circulate and persist in immune populations. However, induction of a strong local T cell response facilitates rapid serum antibody-mediated clearance of *B. pertussis* by overcoming the effects of pertussis toxin. Thus, we propose that novel vaccines to *B. pertussis* would benefit from inducing longstanding Th1 response to *B. pertussis* and serum antibodies to pertussis toxin.

*B. parapertussis*, similar to *B. pertussis*, recently emerged independently from a common *B. bronchiseptica*-like progenitor, and adapted to humans (30, 41). Accordingly, *B. parapertussis* shares high contagiousness, infectivity and pathogenesis with *B. pertussis*. Interestingly, *B. parapertussis* also resists the effect of antibodies but by a mechanism distinct from that of *B. pertussis* (Wolfe D, Kirimanjeswara GS, and Harvill ET, Unpublished data). We hypothesize that *B. parapertussis* has modified its LPS
structure in order to limit inflammation required for the effector functions of antibodies. However, the induction of a T cell or a robust inflammatory response facilitates rapid \textit{B. parapertussis} clearance by antibodies. Therefore, vaccines to \textit{B. parapertussis} may benefit from using adjuvants that induce a robust Th1 response.

\textit{B. bronchiseptica} can efficiently infect a variety of mammals and chronically persist without any symptoms (9). It is intriguing that \textit{B. bronchiseptica} is rarely isolated from immune competent humans in spite of having all the genes present in human adapted bordetellae. Based on our comparative studies, we propose a model for the adaptation of \textit{Bordetella} to different host. \textit{B. bronchiseptica} or its progenitor may have been widely present in human populations. With the emergence of more virulent and contagious \textit{B. pertussis}, the immune response induced against \textit{B. pertussis} may have displaced \textit{B. bronchiseptica} from humans. This theory is supported by our animal studies that indicate that \textit{B. bronchiseptica} is rapidly cleared by serum antibodies and prior infection by \textit{B. pertussis} renders mice immune to subsequent \textit{B. bronchiseptica} challenge. \textit{B. pertussis}, however, was efficient in transiently infecting even an immune individual, and thus persisted in human populations. As a result, \textit{B. parapertussis}, to establish a niche in human population, emerged with an ability to evade immune responses generated against \textit{B. pertussis}. In fact, \textit{B. parapertussis} can successfully infect mice either previously infected and recovered from \textit{B. pertussis} infection or vaccinated for \textit{B. pertussis}. Furthermore, \textit{B. parapertussis} maintains the ability to transiently resist the effect of serum antibodies to persist in an immune population but by a mechanism other than expression of pertussis toxin.
Comparative immunobiology of bordetellae reveals an interesting adaptation strategy of human adapted bordetellae: they have modified their LPS structure in order to induce a minimum inflammatory response. Additionally, \textit{B. pertussis} also has a mechanism, mediated by pertussis toxin, to inhibit inflammation. These strategies perhaps help bacteria to escape innate immune recognition and prolong the infectious period. In addition, bacteria, using the same strategies, are able to evade adaptive immune responses and persist within immune populations. Thus \textit{B. pertussis} and \textit{B. parapertussis} have evolved into successful pathogens that are difficult to eradicate.
REFERENCES


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