Neutralizing Antibodies to Adenylate Cyclase Toxin Promote Phagocytosis of *Bordetella pertussis* by Human Neutrophils

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A previous study showed that opsonization with human immune serum could either promote or antagonize phagocytosis of *Bordetella pertussis* by human neutrophils depending on whether the bacteria expressed adenylate cyclase toxin. Opsonization of the wild-type strain inhibited phagocytosis relative to unopsonized controls. In contrast, mutants lacking adenylate cyclase toxin were efficiently phagocytosed when opsonized with human immune serum. In this study, we examined opsonization in the presence or absence of monoclonal antibodies to adenylate cyclase toxin. Addition of neutralizing monoclonal antibodies to adenylate cyclase toxin converted a serum that previously inhibited both attachment and phagocytosis of the wild-type strain to one that increased both attachment and phagocytosis compared to the no-serum control. Monoclonal antibodies that recognize the adenylate cyclase toxin but fail to neutralize activity were without effect. These results suggest that adenylate cyclase toxin inhibits both Fc receptor-mediated attachment and phagocytosis of *B. pertussis* by neutrophils.

While the current pertussis vaccines protect against severe forms of the disease, many exposed individuals become infected with *Bordetella pertussis* and develop a milder form of the disease (1, 3, 8, 9, 16, 19). Developing pertussis vaccines that prevent bacterial infection is an important goal, since immune responses leading to bacterial elimination would prevent mild forms of the disease, eliminate carriage, and break the cycle of transmission.

Phagocytosis and killing by neutrophils is an effective bacterial defense, but until recently it has not received much attention as an immune defense against pertussis. Using a mouse model, McGuirk and Mills have demonstrated that neutrophils can play a role in immunity to pertussis (14). A significant degree of neutrophil infiltration was observed in the lungs of naive mice and mice immunized with the whole-cell pertussis vaccine following aerosol challenge; however, a neutrophil infiltration was not observed in mice immunized with an acellular pertussis vaccine. Recruitment of neutrophils was correlated with the type of immune response, occurring when Th1 cells were induced in the mice but not when Th2 cells were induced. Unlike mice, humans do not have such a polarized response to pertussis (2, 17, 18), and it is likely that neutrophils would always be recruited during human infection. Harvill et al. demonstrated a role for adenylate cyclase toxin as a bacterial counterdefense to neutrophils (7) when mice were infected with the closely related bacterial pathogen *Bordetella bronchiseptica*. Wild-type organisms, but not adenylate cyclase toxin mutants, caused a lethal infection in T- and B-cell-deficient mice. However, both wild-type and adenylate cyclase toxin mutants were lethal in neutropenic mice, suggesting that neutrophils play a critical role in resolving the infection, and that adenylate cyclase toxin can block clearance by neutrophils (7).

A method was previously developed to quantify phagocytosis using *B. pertussis* labeled with green fluorescent protein (GFP) and differential staining to distinguish extracellular bacteria from phagocytosed, intracellular bacteria (13, 20, 21). Using this technique, a previous study found that 98% of phagocytosed *B. pertussis* bacteria were killed (13); however, wild-type bacteria were phagocytosed very inefficiently by neutrophils, and two virulence factors, filamentous hemagglutinin (FHA) and adenylate cyclase toxin, were shown to influence this process.

Bacterial mutants lacking the adhesin FHA failed to attach to neutrophils and were not phagocytosed. Wild-type strains expressing FHA attached efficiently to neutrophils; however, little phagocytosis was observed. These results suggest that FHA mediates the attachment of *B. pertussis* to neutrophils in a way that fails to provoke phagocytosis. An unexpected result from these studies was that opsonization with an immune serum containing antibodies to FHA (23) caused a significant reduction in bacterial attachment as well as phagocytosis, suggesting that FHA-mediated attachment may be more efficient than Fc-mediated attachment.

Adenylate cyclase toxin also influenced phagocytosis by neutrophils. Adenylate cyclase toxin is an essential virulence factor of *B. pertussis* (5, 22) and has been shown to inhibit the ability of neutrophils to phagocytose and kill microorganisms (4). It is primarily located on the surface of the bacteria (10) and acts as a contact toxin (15). It can elevate intracellular cyclic AMP (cAMP) levels in target cells (this is referred to as adenylate cyclase toxin activity), and it can also cause hemolysis (lysis of red blood cells), an activity that is independent of cAMP generation (6). It was found that in the absence of opsonization, phagocytosis of the adenylate cyclase toxin mutant was very inefficient (21). However, following opsonization with human immune serum, 67% of the adenylate cyclase toxin mutants were internalized, compared to only 5% of the wild-type bacteria. These results suggest that a signal, likely generated when
the antibody binds to the Fc receptors on neutrophils, is needed for \textit{B. pertussis} to be recognized as foreign and phagocytosis to proceed. Adenylate cyclase toxin appears to block this process.

The observation that opsonization with an immune serum could result in reduced phagocytosis of the wild-type strain is disturbing and has important implications for vaccine development. However, the same serum could promote phagocytosis of the adenylate cyclase toxin mutant, and in this study we examined the influence of neutralizing monoclonal antibodies to adenylate cyclase toxin on phagocytosis of wild-type \textit{B. pertussis}. Addition of the monoclonal antibodies alone did not promote phagocytosis. However, neutralizing antibodies to adenylate cyclase toxin promoted phagocytosis of \textit{B. pertussis} opsonized with the immune serum, which by itself inhibited phagocytosis. These studies suggest that adenylate cyclase could be a useful vaccine antigen.

\textbf{Monoclonal antibodies.} Monoclonal antibody preparations described in a previous study were used (12). Antibodies 3D1 and 5D1 are capable of neutralizing adenylate cyclase toxin activity but have no effect on the hemolytic activity of the toxin (12). Antibodies 2A12 and 6E1 can neutralize hemolysin activity but have variable effects on adenylate cyclase toxin activity. Antibody 2A12 inhibits adenylate cyclase toxin activity, but at a much lower potency than 3D1 and 5D1, while antibody 6E1 has no effect on intracellular cAMP levels (12). The antibodies were purified from ascitic fluids and diluted to the same protein concentration (0.88 μg/ml). Enzyme-linked immunosorbent assay titers were 1:1 \times 10^5 for 3D1, 5D1, and 6E1 and 1:5 \times 10^4 for 2A12 (12). All four monoclonals were of the mouse immunoglobulin G1 subtype.

\textbf{Phagocytosis.} Phagocytosis assays using human neutrophils were performed as previously described (13, 21). A variant of BP335 expressing GFP, BP335(pCW504), was used for the phagocytosis studies. Briefly, 10 μl of each monoclonal antibody was added to 3 \times 10^7 bacteria suspended in 30 μl of HBSA (Hanks’ buffer supplemented with 0.25% bovine serum albumin and 2 mM HEPES), followed by the addition of 30 μl of the human immune serum where indicated. The bacteria were incubated at 37°C for 15 min. Following opsonization, bacterial suspensions were adjusted to 400 μl and added to adherent neutrophils (5 \times 10^5). To promote contact, the bacteria were centrifuged onto the adherent neutrophils at 640 \times g for 5 min at room temperature. Phagocytosis was allowed to occur for 1 h at 37°C in 5% CO_2. The cells were washed, and ethidium bromide (100 μg/ml) was added for 15 min at room temperature. Cells were washed and fixed, and phagocytosis was quantified by fluorescence microscopy. Intracellular GFP-labeled bacteria resist staining with ethidium bromide and remain green, while extracellular bacteria accumulate ethidium bromide and appear orange by fluorescence microscopy.

\textbf{Phagocytosis in the presence of monoclonal antibodies to adenylate cyclase toxin.} Opsonization with the mouse monoclonal antibodies alone did not alter phagocytosis or adherence of the wild-type strain, compared to the buffer control (data not shown). A previous study had shown that efficient phagocytosis of the adenylate cyclase toxin mutant occurred only when the bacteria were opsonized with human immune serum (21), and in this experiment the mouse monoclonal antibody would have to serve as the opsonizing antibody. Their failure to promote phagocytosis suggests these antibodies cannot perform this function. The mouse antibodies may not interact optimally with human Fc receptors; alternatively, monoclonal antibodies recognize only one epitope, and very little antibody might be present on the bacterial surface.

\textbf{Phagocytosis in the presence of human immune serum and monoclonal antibodies to adenylate cyclase toxin.} In previous studies (13, 20, 21), it was observed that opsonization with a human immune serum inhibited both attachment and phagocytosis of the wild-type strain, compared to the no-serum control. The same results were obtained in this study. Opsonization inhibited attachment of the wild-type strain about fourfold (Fig. 1), a statistically significant decrease (P < 0.004). Opsonization also inhibited phagocytosis about twofold (Fig. 1), a statistically significant decrease (P < 0.02). Monoclonal antibodies were then added to the bacteria opsonized with the human immune serum (Fig. 1). The addition of monoclonal antibodies 3D1 and 5D1 to the opsonized bacteria caused a statistically significant increase in the number of extracellular bacteria attached to the neutrophils as well as in the number of phagocytosed bacteria relative to the number in opsonized controls (Fig. 1). In contrast, two antibodies, 2A12 and 6E1, did not cause a statistically significant change in either attachment or phagocytosis. Mixing all four antibodies together caused a statistically significant increase in phagocytosis compared to that of the opsonized bacteria (P < 0.03) but was not better than using monoclonal antibodies 3D1 and 5D1 alone.

The adenylate cyclase toxin is a bifunctional toxin (6, 12). It can elevate intracellular cAMP levels in target cells (toxin activity), or it can cause hemolysis, which is independent of cAMP generation (6). The monoclonal antibodies that had been demonstrated previously (12) to be most potent in neutralizing adenylate cyclase toxin activity (3D1 and 5D1) were most effective at promoting phagocytosis (Fig. 1). Antibody 2A12, which also was shown to inhibit adenylate cyclase toxin activity but only at higher concentrations (suggesting a lower potency), did not elicit a statistically significant response in this experiment. Antibody 6E1, which inhibits hemolysin activity but not adenylate cyclase toxin activity, had no effect. These results indicate that antibodies which neutralize adenylate cyclase toxin activity mediate a beneficial effect, but only when combined with human opsonizing antibodies. These data are also consistent with earlier observations that adenylate cyclase...
toxin activity, rather than just enzymatic or hemolytic activity, is required to protect the bacterium from the host defenses (11, 22).

It is becoming clear that *B. pertussis* bacteria are capable of infecting and proliferating in a large proportion of vaccinated individuals. In one study, 33% of the exposed individuals receiving the highly effective five-component acellular pertussis vaccine had evidence of infection and 24% coughed for 21 days or more (19). In the same study, 82% of individuals receiving a licensed whole-cell vaccine had evidence of infection and 65% coughed for 21 days or more. Neutrophils are recruited to the site of an infection whenever inflammatory mediators, such as lipopolysaccharides and formylated peptides (for example, formylmethionyl-Leu-Phe), are present. These bacterial products will be present in a primary infection by *B. pertussis*, and they will also be generated when microbial growth occurs in vaccinated individuals with partial immunity. Since neutrophils are capable of killing *B. pertussis* following phagocytosis (13), activation of this immune response could eliminate bacterial infection. However, our studies suggest that activating this immune response to *B. pertussis* can be complicated, and opsonization with human immune serum can either enhance or antagonize phagocytosis of *B. pertussis* by neutrophils.

Our data are consistent with the model presented in Fig. 2. In the absence of antibodies the bacteria attach to the neutrophils in a way that does not provoke phagocytosis (Fig. 2A). Studies with bacterial mutants deficient in a single virulence factor suggest that adherence is largely, if not entirely, mediated by FHA (21). Interestingly, in the absence of opsonizing antibodies, neither the wild-type strain nor mutants missing the adenylate cyclase toxin are efficiently phagocytosed (21). Similarly, in this study the presence or absence of monoclonal antibodies to adenylate cyclase toxin did not promote phagocytosis when no opsonizing antibody was present. Together these results suggest that when the bacteria attach via FHA, they go largely undetected by the neutrophils.

When the bacteria are opsonized with a human immune serum, attachment can occur by interaction of antibody and Fc receptors (Fig. 2B). However, unlike with most microorganisms, opsonization does not appear to promote phagocytosis of wild-type *B. pertussis*, and the bacteria remain extracellular. In contrast, when the adenylate cyclase toxin activity is absent or diminished by the presence of neutralizing antibodies (Fig. 2C), increased phagocytosis of *B. pertussis* occurs. These results suggest that the elevated levels of cAMP due to adenylate cyclase toxin activity inhibit the signaling that normally occurs following the engagement of the antibody by Fc receptors. Fc receptor signaling causes the neutrophil to undergo cytoskeletal rearrangements resulting in the phagocytosis of the organism, which in turn results in tight association and ultimately internalization (Fig. 2C). It has been shown that *B. pertussis* organisms are killed following phagocytosis (13), suggesting that adenylate cyclase toxin serves as a counterdefense to this potent immune defense.

Adenylate cyclase toxin is not included in any of the current acellular vaccine formulations. Our studies suggest that neutralizing antibodies to adenylate cyclase toxin, in conjunction with opsonizing antibodies, could be beneficial in promoting immunity to *pertussis* by enhancing the phagocytic defense mechanisms.

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