MAJOR ARTICLE

Crucial Role of Antibodies to Pertactin in *Bordetella pertussis* Immunity

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Pertussis, a serious infectious disease of the respiratory tract caused by *Bordetella pertussis*, is reemerging in vaccinated populations. Efforts to curtail this disease are hampered by limited insight into the basis of protective immunity. Opsonophagocytosis was recently found to play a central role in cellular bactericidal activity against *B. pertussis*. In the present study, we studied the specificity of opsonic antibodies. Anti–pertactin antibodies, but not anti–pertussis toxin, anti-fimbriae, or anti–filamentous hemagglutinin antibodies, were found to be crucial for *B. pertussis* phagocytosis. These data are consistent with field studies showing that levels of antibodies to pertactin correlate with protection.

Pertussis, or whooping cough, is a serious disease of the respiratory tract caused by the gram-negative bacterium *Bordetella pertussis*. Despite >40 years of vaccination, pertussis is a disease that occurs in endemic areas, with epidemic outbreaks occurring every 3–5 years. Pertussis ranks fifth, after hepatitis B, measles, tetanus, and *Haemophilus influenzae* type b, as a cause of global mortality, according to vaccine-preventable pediatric infectious diseases [1]. In some vaccinated populations (e.g., in Australia, Canada, The Netherlands, and United States), the incidence of pertussis has been increasing over the last decade [2]. This is exemplified by the situation in The Netherlands. In 1996, the number of reported pertussis cases increased 12-fold, compared with the previous year, and, since then, the incidence of pertussis has remained high [3]. Furthermore, it has become clear that pertussis is not only a childhood disease. Several studies indicated that 12%–26% of adults with persistent cough were infected by *B. pertussis* [4]. Of importance, pertussis in adults is associated with significant morbidity.

Historical data suggest that, initially, the introduction of whole cell–pertussis vaccines in the 1950s significantly reduced disease burden. Although generally effective, whole cell vaccines are reactogenic, causing fever and local reactions. Concerns about the safety of whole cell vaccines led to the development of acellular vaccines, composed of ⩾1 of the following *B. pertussis* proteins: pertussis toxin (Ptx), filamentous hemagglutinin (FHA), pertactin (Prn), and serotype 2 and 3 fimbriae (Fim2 and Fim3). Acellular vaccines have now been introduced in a number of countries, replacing whole cell vaccines. As yet, it is unclear whether acellular vaccines will prove more effective than whole cell vaccines in the long run.

Lack of insight into the basis of protective immunity against disease and infection has hampered efforts to improve pertussis vaccines. Two studies indicated that persons with high levels of antibodies to Prn, Fim2, Fim3, or Ptx are less likely to develop clinical disease when exposed to pertussis [5, 6]. However, the mechanism of antibody-mediated protection is poorly understood. The present study provides a rational basis

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for the observed correlation between protection against pertussis and antibodies to Prn and shows that such antibodies are crucial for phagocytosis of *B. pertussis* by immune cells.

MATERIALS AND METHODS

Bacterial strains. *B. pertussis* B213 and B1686 are Tohama streptomycin-resistant derivatives. Strain B1686 does not produce pertactin because of a knockout mutation. The strains were transformed with plasmid pCW505 [7] (supplied by Dr. A. A. Weiss, Cincinnati), which induces cytoplasmic expression of green fluorescent protein. Bacteria were stored at −70°C, recovered by growth on Bordet-Gengou agar plates at 35°C for 3 days, and subsequently recultured overnight on Bordet-Gengou plates before use in phagocytosis experiments.

Serum samples. Serum samples from a study with pertussis vaccines in Dutch children aged 4 years [8] were used in this study. Children were vaccinated at the ages of 3, 4, 5, and 11 months with the Dutch whole cell vaccine and were boosted with an acellular vaccine consisting of FHA, Ptx, and Prn (GlaxoSmithKline) at the age of 4 years. Before and after booster vaccination, serum agglutination activity and titers of specific antibody to Ptx, FHA, Prn, Fim2, and Fim3 were determined (table 1). Written informed consent was obtained from a parent, guardian, or adult before enrollment. This study was approved by the institutional review board of the National Institute of Public Health and the Environment, The Netherlands.

Peripheral blood polymorphonuclear leukocytes (PMNLs). Peripheral blood PMNLs were isolated from heparinized venous blood of healthy human volunteers (age range, 25–45 years) by Histopaque (Sigma-Aldrich) gradient centrifugation. PMNLs were harvested, and the remaining erythrocytes were removed by hypotonic lysis. Cell viability was >99%, as determined by trypan blue exclusion. Before functional assays, PMNLs were washed twice with RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, resuspended, and used immediately. All experiments described in this study were conducted with freshly isolated PMNLs lacking Fcγ receptor type I expression, as monitored by cell cytometric analyses with fluorescein isothiocyanate–conjugated anti-Fcγ receptor type I monoclonal antibody 22 [9].

Phagocytosis. Phagocytosis of *B. pertussis* was evaluated as described elsewhere [10]. In brief, green fluorescent protein–expressing *B. pertussis* were grown overnight on Bordet-Gengou agar plates and resuspended in RPMI 1640 medium containing 10% fetal calf serum. Bacteria were incubated with or without 5% human serum for 30 min at 37°C. After being washed, opsonized and nonopsonized bacteria were incubated with phagocytic cells in a 70:1 ratio for 45 min at 4°C, to allow for binding of bacteria to PMNLs. After extensive washing to remove nonattached bacteria, cells were split into 2 aliquots and were further incubated for 30 min at either 4°C or 37°C. Next, remaining cell surface–bound opsonized bacteria were detected by incubation (30 min at 4°C) with phycoerythrin-conjugated goat $F(ab')_2$ of anti–human IgG (Southern Biotechnology). After washing, samples were analyzed by flow cytometry. Five thousand cells per sample were analyzed. Green and red fluorescence intensities of cells maintained at 4°C throughout served as controls for bacterial binding (i.e., no phagocytosis). The decrease in red fluorescence of green-positive cells after incubation at 37°C reflects bacterial phagocytosis, as confirmed microscopically [10]. Phagocytosis rates were calculated from the drop in mean red fluorescence intensity of green-positive cells, as described elsewhere [10].

Table 1. Titers of antibodies to pertussis toxin (Ptx), filamentous hemagglutinin (FHA), pertactin (Prn), and type 2 and 3 fimbriae (Fim2 and Fim3), and agglutinating activity of serum samples from donors after booster vaccination.

Donor	Virulence factor					Agglutinating activity
	Ptx	FHA	Prn	Fim2	Fim3	
1	30	199	33	12	5	4
2	81	66	26	43	9	32
3	8	107	78	57	13	4
4	69	1293	154	23	9	4
5	83	138	120	69	63	8
6	109	2148	156	136	96	128
7	98	232	56	94	12	64
8	24	48	518	45	3	16
9	826	286	88	160	15	2560
10	1411	430	156	212	75	256
11	496	109	517	158	24	64
12	148	981	305	76	17	32
13	1065	2232	454	457	165	256
14	68	222	349	64	10	32
15	3	109	333	240	85.5	512
16	16	607	828	96	5	32

NOTE. Data are antibody titers (ELISA units/mL calculated relative to the US reference pertussis antiserum).

ELISA and bacterial agglutination. Titers of antibodies to Ptx, FHA, Prn, Fim2, and Fim3 were determined by ELISA, as described elsewhere [11]. In brief, 96-well plates were coated with purified Ptx, FHA, Fim2, Fim3 (National Institute of Health and the Environment), or Prn (Chiron) at a concentration of 1, 2, 3, 3, and 3 μg/mL, respectively. For each serum, eight 2-fold dilutions were tested starting with a 1:60 dilution. Bound IgG was detected after 2 h of incubation (room temperature) with alkaline phosphatase–

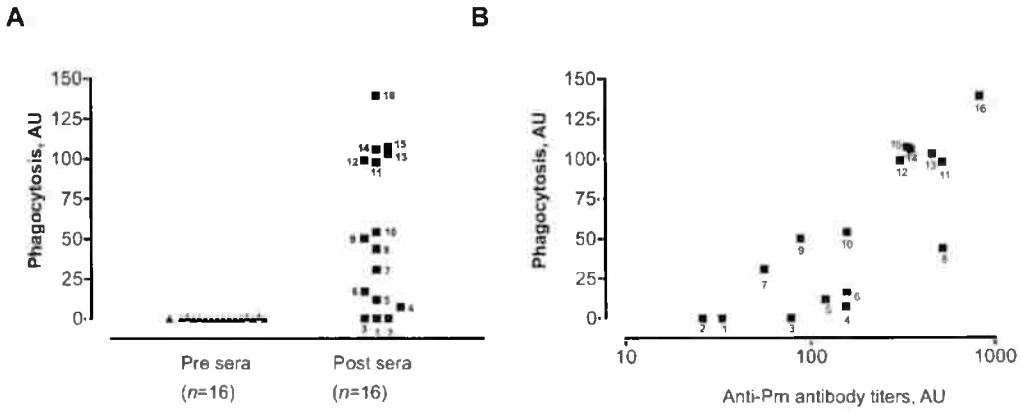

Figure 1. Effect of serum samples collected before and after booster vaccination on phagocytosis by human polymorphonuclear leukocytes (PMNLs). *A, Bordetella pertussis* were opsonized with human sera (5%) obtained before (Pre sera) or after (Post sera) booster vaccination with a 3-component acellular vaccine consisting of filamentous hemagglutinin, pertussis toxin, and pertactin. Phagocytosis by freshly isolated human PMNLs was expressed in arbitrary units (AU). Data from 1 experiment of 3 are shown. *B,* Correlation between *B. pertussis* phagocytosis and anti–pertactin antibody titers in serum samples obtained after booster vaccination. Serum nos. in figure correspond with serum nos. in table 1.

conjugated goat anti–human IgG (Sigma; working dilution, 30,000).

ELISA units (EU) were calculated relative to the US reference pertussis antiserum (human; obtained from the US Food and Drug Administration), lot 3 for Ptx, FHA, and Fim and lot 4 for Prn, by use of the 4-parameter fit method in KC4 (Kineticalc for Windows) with a BioTek plate reader (EL312e; BioTek). The minimum level of detection was estimated to be 2 EU/mL for anti–Ptx and anti–FHA antibodies, 4 EU/mL for anti–Prn antibodies, 2 EU/mL for anti–Fim2 antibodies, and 1 EU/mL for anti-Fim3 antibodies.

Agglutinating antibodies were measured essentially as described elsewhere [12], with use of *B. pertussis* 3838 as antigen. US pertussis antiserum was used as reference, and a control serum was included on each plate. For each serum, a 2-fold dilution series in 8 wells was tested (starting with a 1:4 dilution). The agglutination titer is expressed as the reciprocal of the highest final dilution of serum resulting in agglutination.

Statistical analysis. Antibody titers were converted to $\log_{10}$ values before analyses for linear correlation. Pearson's product moment correlation coefficient [13] was calculated for phagocytosis rates and the inverse of log-converted titers of antibody to selected bacterial antigens. Student's *t* test was used to assess the significance of differences between phagocytosis rates induced by the different sera. Significance was accepted at $P < .05$.

RESULTS

B. pertussis containing pCW505, a plasmid coding for the green fluorescent protein, was incubated with serum specimens from 16 children obtained before and after booster vaccination with acellular vaccine. Subsequently, bacteria were incubated with human PMNLs, and phagocytosis was quantified by flow cytometry. Thirteen of the 16 postvaccination serum samples enhanced phagocytosis. In contrast, no phagocytic effect was observed and no antibodies were detected by ELISA or agglutination in any of the 16 serum samples obtained before booster vaccination (figure 1*A*). A statistically significant correlation was found between phagocytosis and titers of antibody to Prn ($R^2_{Prn} = 0.71$ and $P_{Prn} = .001$; figure 1*B*), whereas correlations with agglutination titers ($R^2_{agg} = 0.01$ and $P_{agg} = .9$) or titers of antibody to Ptx, FHA, Fim2, and Fim3 were not significant (respectively, $R^2_{Ptx} = 0.32$ and $P_{Ptx} = .13$; $R^2_{FHA} = 0.03$ and $P_{FHA} = .17$; $R^2_{Fim2} = 0.2$ and $P_{Fim2} = .09$; and $R^2_{Fim3} = 0.05$ and $P_{Fim3} = .4$).

To further investigate the role of antibodies to Prn in inducing phagocytosis, 3 serum samples with different levels of anti–pertactin antibodies (donors 9, 13, and 16) were depleted of antibodies to Prn, Fim2, or Fim3 by incubation with the respective purified proteins. Purity of Prn and Fim antigens used for depletion was confirmed by SDS-PAGE and immunoblot analyses. After depletion of antibodies to Prn, the ability of the serum to induce phagocytosis was nearly absent, whereas depletion of antibodies to Fim2 and Fim3 had no such effect (figure 2*A*). Of importance, although a drastic decrease in anti–Prn antibody titer was observed after incubation with purified Prn, neither titers of antibodies to the other antigens (Ptx, FHA, Fim2, and Fim3) nor serum agglutinating activity were affected (data not shown). Similarly, incubation of serum samples with Fim2 and Fim3 antigens solely depleted anti–Fim2 and anti–Fim3 antibodies from serum, as determined by ELISA. Finally, serum samples that were potent in facilitating phagocytosis exhibited a strongly reduced ability to induce phagocytosis of a *B. pertussis* strain defective in Prn expression (figure 2*B*). In combination, these data show Prn to represent an important target for antibody-mediated phagocytosis.

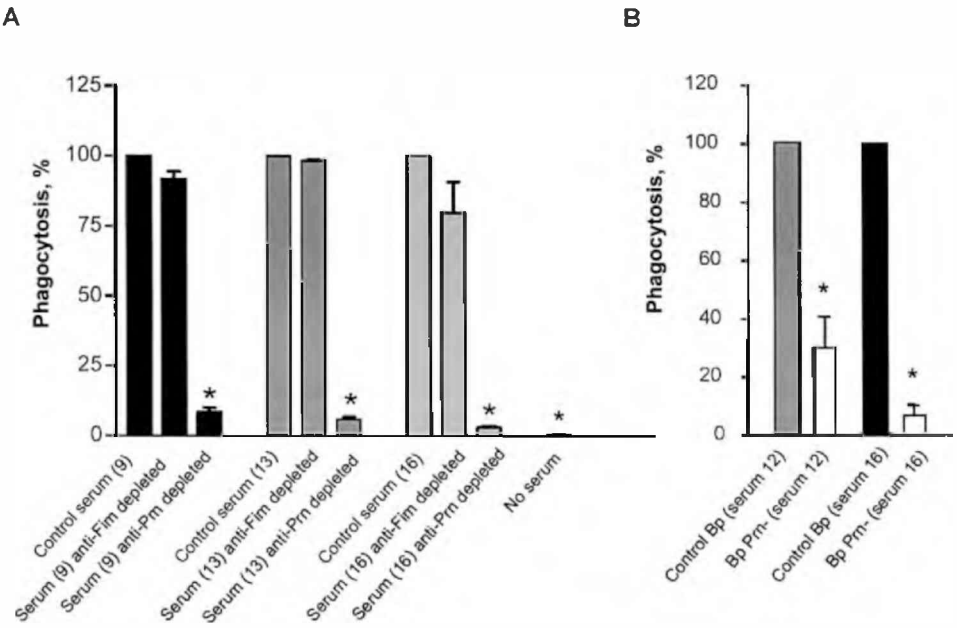

Figure 2. Antibodies to pertactin are crucial for phagocytosis. *A,* Serum samples collected after booster vaccination (Post sera) were either depleted of antibodies directed against serotype 2 and 3 fimbriae (Fim) or pertactin (Prn) by overnight incubation with saline (Control serum) or the respective purified proteins. Subsequently, *Bordetella pertussis* was incubated with and without the respective serum sample, and phagocytosis was quantified. Phagocytosis induced by control serum was set at 100%. *Phagocytosis induced in the absence of serum, or by anti–Prn antibody–depleted serum, was statistically different from phagocytosis induced by control serum or anti–Fim antibody–depleted serum ($P < .05$; 2-sided Student's *t* test). *B,* Wild-type *B. pertussis* (Bp) and a *B. pertussis* mutant without Prn expression (Bp Prn−) were opsonized with postvaccination serum (serum 12 and serum 16), and phagocytosis was quantified. Phagocytosis of the wild-type strains was arbitrarily set at 100%. *$P < .05$, between phagocytosis values of opsonized wild-type *B. pertussis* strain and opsonized *B. pertussis* Prn− mutant strain. Data are mean ± SD of 3 independent experiments.

DISCUSSION

Previous studies have indicated that antibodies to Prn are important for protection against disease. Field trials revealed clinical protection to correlate with levels of anti–Prn antibodies [5, 6]. Furthermore, 3-component acellular vaccines containing FHA, Ptx, and Prn were shown to be significantly more efficacious than 2-component vaccines with solely FHA and Ptx in the same study (vaccine efficacies of 84% vs. 59%) [14]. Prn is one of the few *B. pertussis* proteins that exhibit antigenic variation [15], and antigenic divergence between vaccine strains and clinical isolates has been observed in a number of vaccinated populations, which suggests a significant effect of anti–Prn antibodies on strain transmission [16–19]. By use of a mouse model, it was shown that the variable region of Prn harbors a protective epitope and that a whole cell vaccine was less effective against strains producing nonvaccine-type Prn variants [20]. Phagocytosis and subsequent killing of microorganisms represents a potent immune mechanism for clearing bacteria from the respiratory tracts of infected persons. We have recently demonstrated that antibody-mediated phagocytosis is a key mechanism for *B. pertussis* killing by phagocytes [9, 21]. By using a flow cytometry-based phagocytosis assay that allows accurate assessment of *B. pertussis* phagocytosis, we could determine that, in the absence of opsonic antibodies, binding of *B. pertussis* to phagocytes is drastically reduced. Under these conditions, neither bacterial phagocytosis nor cellular bactericidal effector functions against this pathogen takes place. In the present study, anti–Prn antibodies, but not anti–Ptx, anti–Fim2, anti–Fim3, or anti–FHA antibodies, were shown to be crucial for *B. pertussis* phagocytosis. These data provide a biological basis for the well-documented, but poorly understood, observation of a close correlation between antibodies to Prn and protection. In contrast to anti–FHA antibodies, antibodies to Ptx, Fim2, and Fim3 also were found to correlate with protection in humans [5, 6]. Although we found no evidence for involvement of antibodies to Ptx, Fim2, and Fim3 in phagocytosis, these antibodies may well confer protection by other mechanisms, such as complement-mediated killing, blocking of bacterial adherence, and antitoxin activity. Prn is the only antigen present in acellular vaccine that induces opsonic antibodies. Prn variants have been documented in a number of populations over the years and are presently causing outbreaks [15–19]. This study provides a new view on the importance of Prn as a vaccine candidate despite the traditional view of this antigen as a mere adhesin to be blocked. Moreover,

it provides new and relevant evidence that sheds some light on the problem of *B. pertussis* persistence within the population.

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