Role of Antibodies against *Bordetella pertussis* Virulence Factors in Adherence of *Bordetella pertussis* and *Bordetella parapertussis* to Human Bronchial Epithelial cells

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Immunization with whole-cell pertussis vaccines (WCV) containing heat-killed *Bordetella pertussis* cells and with acellular vaccines containing genetically or chemically detoxified pertussis toxin (PT) in combination with filamentous hemagglutinin (FHA), pertactin (Prn), or fimbriae confers protection in humans and animals against *B. pertussis* infection. In an earlier study we demonstrated that FHA is involved in the adherence of these bacteria to human bronchial epithelial cells. In the present study we investigated whether mouse antibodies directed against *B. pertussis* FHA, PTg, Prn, and fimbriae, or against two other surface molecules, lipopolysaccharide (LPS) and the 40-kDa outer membrane porin protein (OMP), that are not involved in bacterial adherence, were able to block adherence of *B. pertussis* and *B. parapertussis* to human bronchial epithelial cells. All antibodies studied inhibited the adherence of *B. pertussis* to these epithelial cells and were equally effective in this respect. Only antibodies against LPS and 40-kDa OMP affected the adherence of *B. parapertussis* to epithelial cells. We conclude that antibodies which recognize surface structures on *B. pertussis* or on *B. parapertussis* can inhibit adherence of the bacteria to bronchial epithelial cells, irrespective whether these structures play a role in adherence of the bacteria to these cells.

*Bordetella pertussis* is the major causative agent of whooping cough (pertussis), a highly contagious infection of the respiratory tract in humans. To establish efficient colonization of the respiratory tract, this gram-negative coccobacillus produces a variety of virulence factors that contribute to its adherence to the respiratory epithelium. Recently we described a role for the bacterial virulence factors filamentous hemagglutinin (FHA) and fimbriae in the adherence of *B. pertussis* to two kinds of epithelial cells of the human respiratory tract (39). Other virulence factors such as pertussis toxin (PT) and pertactin (Prn) were not involved in the adhesion of *B. pertussis* to these human epithelial cells (39). Studies in mice have shown that immunization with purified *B. pertussis* FHA (34, 43), PT (9, 26, 37), fimbriae (16, 18, 35, 41, 43), or Prn (9, 34) protects against an intranasal or aerosol challenge with *B. pertussis*. In humans, the presence of antibodies against FHA and fimbriae also seems to correlate with protection against *B. pertussis* infection and the incidence of whooping cough (4, 6, 14, 24). Together, these studies may imply that antibodies against *B. pertussis* virulence factors interfere with adherence of the bacteria to the respiratory tract epithelium.

*Bordetella parapertussis*, a bacterium closely related to *B. pertussis*, also causes pertussis-like symptoms in humans (15, 19, 21, 22, 27, 28, 38). *B. parapertussis* does not produce PT, but most other virulence factors produced by *B. parapertussis*, including FHA, fimbriae, and Prn, are homologous to those produced by *B. pertussis* (1). Various clinical studies, however, found that vaccination with whole-cell pertussis vaccines (WCV) or even infection with *B. pertussis* does not protect against infection with *B. parapertussis* (7, 10, 18, 19, 27). Thus, despite the high degree of homology of virulence factors between *B. pertussis* and *B. parapertussis*, antibodies against *B. pertussis* do not prevent *B. parapertussis* colonization. This finding was confirmed by animal studies which showed limited or no cross-protection against *B. parapertussis* (18, 41).

In most countries, protection against whooping cough is based on the use of WCV containing heat-killed *B. pertussis*. Alternatively, acellular vaccines with various combinations of purified and detoxified PT and other *B. pertussis* virulence factors, such as FHA, Prn, and fimbriae, have been developed and in some countries used instead of WCV. However, it is not known how antibodies induced by components of acellular vaccines confer protection and to what extent they also protect against *B. parapertussis*.

In the present study, we investigated whether antibodies elicited in mice against purified *B. pertussis* virulence factors affected the adherence of *B. pertussis* to the human bronchial epithelial cell line NCI-H292. Antibodies against WCV served as controls. Furthermore, we studied whether these antibodies cross-reacted with *B. parapertussis* and affected the adherence of the bacteria to bronchial epithelial cells as well.

MATERIALS AND METHODS

**Bacteria and purified bacterial proteins.** Strains used in this study were *B. pertussis* Tohama I (36) and *B. parapertussis* B24 (25), both human clinical isolates. The *B. parapertussis* isolate is a typical strain as determined by serology at the National Institute of Public Health and the Environment (Bilthoven, The Netherlands). Bacteria were cultured for 2 days on Bordet-Gengou agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 15% sheep blood. Before use, bacteria were harvested and suspended in phosphate-buffered saline (PBS; pH 7.4). The number of bacteria was determined with a spectrophotometer at 600 nm and then adjusted to 109 CFU/ml in HAP medium (PBS containing 3 mM glucose, 150 mM CaCl2, 500 mM MgCl2, 0.3 U of aprotinin per ml, and 0.05% [wt/vol] human serum albumin). The number of bacteria was confirmed by colony counts after plating on Bordet-Gengou agar.

Purified native *B. pertussis* fimbriae used in this study were kindly provided by A. Robinson (Centre for Applied Microbiology & Research, Porton Down, United Kingdom); purified native *B. pertussis* FHA and Prn and genetically detoxified PT (PTg) were kindly provided by R. Rappuoli (Biocine SpA, Siena, United Kingdom); and purified native *B. parapertussis* FHA and Prn were kindly provided by A. Robinson (Centre for Applied Microbiology & Research, Porton Down, United Kingdom).
TABLE 1. Antigen-specific antibody titers and immunoglobulin concentrations in mouse serum against various B. pertussis virulence factors or WCV

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean antigen-specific antibody titer (log_{10} ± SD)</th>
<th>Mean concn (μg/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgA</td>
</tr>
<tr>
<td>Normal mouse serum</td>
<td>196 ± 14</td>
<td>21 ± 18</td>
</tr>
<tr>
<td>Serum of mice immunized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FHA</td>
<td>6.1 ± 0.35</td>
<td>3,340 ± 420</td>
</tr>
<tr>
<td>PTg</td>
<td>5.7 ± 0.37</td>
<td>3,162 ± 354</td>
</tr>
<tr>
<td>Fimbriate</td>
<td>6.0 ± 0.89</td>
<td>3,162 ± 382</td>
</tr>
<tr>
<td>Pn</td>
<td>6.1 ± 0.85</td>
<td>4,639 ± 402</td>
</tr>
<tr>
<td>WCV</td>
<td>6.0 ± 0.32</td>
<td>3,180 ± 354</td>
</tr>
</tbody>
</table>

a Mice were immunized as described in Materials and Methods. Pooled serum from 10 mice was used, and antigen-specific antibody titers and concentrations of total immunoglobulins were detected by ELISA.

b Specific for homologous antigens in ELISA; reciprocal of the highest dilution corresponding with three times the blank value, ND, not determined.

c WCV used as antigen in ELISA.

Italy). WCV and tetanus toxoid (TT) were obtained from the National Institute of Public Health and the Environment.

FITC labeling of bacteria. B. pertussis and B. parapertussis were labeled with fluorescein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, Mo.) as described previously (13, 42). Briefly, bacteria (10^8/ml) were incubated in a solution of 1 mg of FITC per ml, 50 mM sodium carbonate, and 100 mM NaCl (pH 9.0) for 20 min at room temperature, washed four times to remove excess FITC, and resuspended in HAP medium to a final concentration of 10^9/ml. The bacteria were kept for 30 min at 37°C until use. This procedure had no effect on either the viability of the bacteria or the binding sites of virulence factors involved in adherence of B. pertussis to epithelial cells (39).

Cells. The human bronchial epithelial cell line NCI-H292 (CRL-1888; American Tissue Culture Collection, Rockville, Md.) was used. The cells were cultured in RPMI 1640 (Gibco, Grand Island, N.Y.) containing sodium penicillin (100 U/ml), streptomycin (50 μg/ml), 2 mM l-glutamine, and 10% heat-inactivated fetal calf serum (Gibco) in uncoated tissue culture flasks (Greiner Labortecnik, Frickenhausen, Germany). Before use in the adherence assay, NCI-H292 cells were detached with 1 mM EDTA in PBS at 37°C for 5 min and washed, and 5 x 10^5 cells per well in protein-free medium (Ultradoma-PF; Boehringer Ingelheim/BioWhittaker, Verviers, France) supplemented with sodium penicillin G (1,000 U/ml) and streptomycin (50 μg/ml) were cultured overnight on Terasaki plates (Greiner Labortecnik).

Preparation of mouse sera against B. pertussis virulence factors. Specific-pathogen-free mice (BALB/c/RIVM) were used and kept in protective isolators. The mice were routinely checked according to standard operating protocols at the National Institute of Public Health and the Environment for infection with a large number of pathogens, including gram-negative bacteria such as Bordetella bronchiseptica, Klebsiella pneumoniae, members of the family Enterococcaceae, Pseudomonas aeruginosa, Salmonella sp., and Yersinia enterocolitica. On days 0 and 28, mice were immunized intraperitoneally with 5 μg of purified B. pertussis FHA, PTg, fimbriae, or Pn, each adsorbed to aluminum hydroxide (25% Alu-Gel-S; Seradyn, Germany). In PBS. Control mice were immunized with TT (5 μg/ml) adsorbed to aluminum hydroxide (25% Alu-Gel-S) in PBS or with aluminum hydroxide (25% Alu-Gel-S) in PBS. Two weeks after the second immunization, sera of 10 mice were collected and pooled.

In addition, mice were immunized subcutaneously at days 0 and 14 with 3.2 μg of purified FHA, PTg, fimbriae, or Prn per ml in 50 mM sodium carbonate buffer (pH 9.6) at 37°C. The various subclasses were detected using corresponding peroxidase-conjugated goat anti-mouse IgG, IgA, or IgM (SBA). The total concentration of immunoglobulin subclasses in mouse sera were determined by using purified IgG, IgA, or IgM (SBA) as the standard.

Binding of Mabs to B. pertussis and B. parapertussis was determined by ELISA as described for antibodies in mouse serum except that the plates were coated overnight with 5 x 10^6 heat-killed B. pertussis or B. parapertussis per ml suspended in a 50 mM sodium carbonate buffer (pH 9.6) at 37°C. Values of endpoint titration curves are given as the reciprocal of the highest dilution corresponding with three times the blank value and expressed as log_{10}.

Adherence assay. Adherence of the bacteria to the surface of cultured NCI-H292 cells was as assessed as described previously (39), with some minor modifications. Overnight cultures on Terasaki plates containing 5 x 10^5 cells per well were washed three times with warm PBS. Next, 3 x 10^4 FITC-labeled bacteria in HAP medium were added to each well and incubated in the presence of various polyclonal or monoclonal antibodies for 45 min at 37°C. After five washes with warm PBS to remove nonadherent bacteria, the plates were fixed for 15 min with 0.05% glutaraldehyde (Polyscience Inc., Warrington, Pa.). After two additional washes with PBS at room temperature, the plates were examined with fluorescence microscopy at a magnification of x400. The number of bacteria adherent to 100 cells was determined. In other experiments, B. pertussis and B. parapertussis were preincubated with 1% mouse immune serum for 30 min at 37°C before three washes, the binding of bacteria coated with antisera to epithelial cells was assessed as described above. All immune sera and Mabs used did not agglutinate the bacteria in the concentrations used in the different assays (data not shown).

Statistical analysis. Differences between the results of the various experiments were evaluated by means of analysis of variance (ANOVA) and Newman-Keuls multiple-comparison test.

RESULTS

Antibody response against B. pertussis virulence factors or B. pertussis WCV. Sera of mice immunized with purified B. pertussis virulence factors FHA, PTg, fimbriae, and Pn showed similar titers of antigen-specific antibodies (Table 1); no cross-reacting antibodies were found against the other purified components (data not shown). In sera of mice immunized with WCV, the antibody titer for WCV used as antigen was comparable to the titers of antigen-specific antibodies in sera of mice immunized with purified virulence factors (Table 1). In the sera obtained after WCV vaccination, the antibody titers...
against FHA, Prn, and fimbriae were $-\log_{10} 5.8$, $-\log_{10} 5.4$, and $-\log_{10} 4.5$, respectively; no antibody against PTg was detected. Sera of immunized mice contained considerably higher amounts of IgG and IgM, but not of IgA, compared to normal mouse serum (Table 1).

**Inhibition of adherence of** B. pertussis **to NCI-H292 cells by anti-B. pertussis mouse sera.** The effect of anti-B. pertussis antibodies on the adherence of B. pertussis to NCI-H292 cells was studied by incubation of bacteria with epithelial cells in the presence of immune sera or anti-TT serum, which served as a control. Immune serum against FHA, PTg, fimbriae, Prn, or WCV reduced the adherence of B. pertussis to NCI-H292 cells (Fig. 1). The inhibition of adherence was concentration dependent and reached significance ($P < 0.05$) with 2.5% serum in comparison to the same concentration of anti-TT serum. Both anti-TT serum (Fig. 1) and normal mouse serum (data not shown) also reduced adherence of B. pertussis to epithelial cells, although not significantly.

For convenience, the effect of antibodies on the adherence of B. pertussis is expressed as the number of B. pertussis to 100 epithelial cells (Fig. 1). However, this value is derived from the change in the percentage of positive epithelial cells and the number of B. pertussis organisms per positive epithelial cell (Table 2). The results showed that in the presence of 2.5% serum containing antibodies against virulence factors, the percentage of positive cells and the number of B. pertussis organisms per positive cell are lower than in the absence of serum (HAP medium) or anti-TT serum.

**Adherence of B. parapertussis to NCI-H292 cells in the presence anti-B. pertussis mouse sera.** In the absence of serum, adhesion to bronchial epithelial cells of B. parapertussis (Fig. 2) was less than that of B. pertussis (Fig. 1), being $73 \pm 13$ (mean $\pm$ standard deviation [SD]) and $96 \pm 29$ bacteria/100 epithelial cells, respectively. Antiserum against B. pertussis FHA, PTg, fimbriae, Prn, or WCV did not significantly reduce the adherence of B. parapertussis to the epithelial cells compared to anti-TT serum (Fig. 2). With all mouse sera, including anti-TT serum and normal mouse serum (data not shown), there was a reduced binding of B. parapertussis to NCI-H292 cells, and this effect became greater with increasing concentrations of serum (Fig. 2).

**Adherence of B. pertussis or B. parapertussis preincubated with anti-B. pertussis serum to NCI-H292 cells.** The above-described experiments showed a reduced although not significantly so, adherence of B. pertussis and B. parapertussis to epithelial cells in the presence of anti-TT serum, which was used as a control (Fig. 1 and 2). To examine whether serum factors other than antibodies bound to B. pertussis or B. parapertussis play a role in inhibiting adherence of these bacteria, the equivalent concentration of anti-TT serum was determined by ANOVA and Newman-Keuls multiple-comparison test: *, $P < 0.05$ versus anti-TT serum.

![Image](http://iai.asm.org/)
the bacteria were preincubated with 1% antiserum against *B. pertussis* FHA, PTg, fimbriae, Prn, WCV, or TT, or with HAP medium lacking serum, and next incubated with NCI-H^292^ cells. Preincubation of *B. pertussis* with antiserum against the various *B. pertussis* virulence factors was found to lead to a 40 to 60% reduction in adherence compared to preincubation with anti-TT serum, which did not affect adherence of bacteria to epithelial cells (Table 3). Preincubation of *B. parapertussis* with these specific antisera did not affect the adherence of this microorganism (Table 3). These results indicate that the inhibition of binding observed in both immune and anti-TT sera, was not due to binding of serum components other than antibodies to the bacterial surface.

### Adherence of *B. pertussis* or *B. parapertussis* to NCI-H^292^ cells in the presence of MAb against *B. pertussis* FHA, LPS, or 40-kDa OMP.

Antibodies against the various virulence factors of *B. pertussis* were equally effective in reducing the adherence of these bacteria to epithelial cells. Since these antisera were used in nonagglutinating concentrations (data not shown), the question arose as to whether the observed effect was due either to blocking of the interaction of the adhesin with its receptor or to steric hindrance. Both LPS and the 40-kDa OMP are abundantly present on the surface of virulent- as well as avirulent-phase *B. pertussis* and *B. parapertussis* (2, 3, 8, 11, 29), but these surface antigens are not implicated in the adherence of *B. pertussis* to respiratory epithelial cells (39). Using an ELISA technique, we found that both *B. pertussis* and *B. parapertussis* bound MAb against LPS or 40-kDa OMP, whereas *B. pertussis* but not *B. parapertussis* bound MAb against FHA (Table 4). Adherence of *B. pertussis* to epithelial cells in the presence of MAb against FHA, LPS, or 40-kDa OMP was significantly lower than in the presence of HAP medium; adherence of *B. parapertussis* in the presence of MAb against LPS or 40-kDa OMP was also significantly reduced, but MAb against FHA had no such effect (Fig. 3).

### DISCUSSION

The major conclusions of this study are that antibodies against the *B. pertussis* virulence factors FHA, PTg, fimbriae,
and Prn inhibited adherence of *B. pertussis* but not of *B. parapertussis* to human bronchial epithelial cells. The adherence of both *B. pertussis* and *B. parapertussis* was inhibited by antibodies against LPS and the 40-kDa OMP of *B. pertussis*.

Various reports have shown in a murine infection model complementary roles for humoral and cell-mediated immunity in the protection against *B. pertussis* (23, 30, 33). In these publications, it has been suggested that cell-mediated immunity against intracellular *B. pertussis* provides optimum protection and rapid elimination of bacteria from the lungs. However, another important function of cellular immunity is the regulation of antibody production by T cells, which is necessary for limiting the infection by preventing initial bacterial adherence to respiratory epithelial cells, neutralization of bacterial toxins, and optimal removal of extracellular bacteria through opsonization (23).

Our results, which showed that antibodies raised against the *B. pertussis* virulence factors FHA, PTg, fimbriae, and Prn reduced the adherence of *B. pertussis* to epithelial cells, are in agreement with the protective role of antibodies for a *B. pertussis* infection in mice, immunized with either FHA (9, 34, 43), PT (9, 26, 37), fimbriae (16, 17, 35, 41, 43), or Prn (9, 34). In addition, the reduced adherence of *B. pertussis* to epithelial cells may indicate that such antibodies present in serum of children vaccinated with WCV or recovered from whooping cough (4, 6, 14, 24) are relevant for the protection against a *B. pertussis* infection as well.

We found that antisera against individual virulence factors were equally effective in inhibiting adherence of *B. pertussis* to bronchial epithelial cells. Furthermore, antiserum against WCV, which contains antibodies against, among others, FHA, fimbriae, and Prn, was not more effective in reducing the adherence of *B. pertussis* to epithelial cells. Analysis of our data showed that not only the number of *B. pertussis* organisms per positive bronchial epithelial cell but also the percentage of positive epithelial cells was reduced. Since all sera contained comparable antigen-specific antibody titers and the concentrations of total immunoglobulins were similar, our data suggest that the combinations of antibodies against the various factors present in antiserum against WCV act additively in inhibiting adherence.

In another study, we demonstrated that only FHA is involved in the adherence of *B. pertussis* to bronchial epithelial cells (39). Since antibodies against PTg, Prn, and fimbriae, which are not involved in the adherence of *B. pertussis* to bronchial epithelial cells (39), and even antibodies against LPS and the 40-kDa OMP reduced the adherence of *B. pertussis* to these epithelial cells, our results indicate that antibodies against surface structures of *B. pertussis* other than adhesion factors can interfere with bacterial adherence.

Anti-TT serum or normal mouse serum also reduced the adherence of *B. pertussis* and *B. parapertussis* to epithelial cells, which indicates that under the experimental conditions used, serum factors other than antibodies against virulence factors interfere with adherence. Fibronectin, which is a major serum component, may account for this effect, since in a preliminary experiment the adherence of *B. pertussis* to bronchial epithelial cells was inhibited about 44% by the presence of fibronectin. An equivalent concentration of collagen had no such effect. Preincubation of *B. pertussis* and *B. parapertussis* with anti-TT serum did not reduce the adherence of these bacteria to bronchial epithelial cells, which suggests that fibronectin may block the host receptors and thus prevent adherence of the bacteria. In this regard, it is interesting that fibronectin and fimbriae of *B. pertussis* can bind to similar receptors and have similar binding specificities (12).

The adherence of *B. parapertussis* to bronchial epithelial cells was not inhibited by antibodies against *B. pertussis* virulence factors, although a nonsignificant effect was observed in the presence of 2.5% anti-FHA serum. This may explain why mice immunized with purified pertussis toxoid, FHA, or Prn are not protected against infection with *B. parapertussis* (18), although some protection against *B. parapertussis* was obtained by immunization with WCV or purified fimbriae (41).

MAbs against *B. pertussis* LPS and the 40-kDa OMP bound to both *B. pertussis* and *B. parapertussis* and inhibited their adherence to epithelial cells. These data suggest that LPS, which contains very conserved regions located at the proximal and intermediate regions near the lipid A part (8, 20), may elicit antibodies that are cross-protective between the two *Bordetella* species. This is in agreement with the finding that the 40-kDa OMP, which is also very conserved between various *Bordetella* species (2, 3), can elicit cross-protective antibodies after appropriate presentation (32). However, antiserum from WCV-immunized mice, which most likely also contains antibodies against LPS and 40-kDa OMP, did not reduce adherence of *B. parapertussis* to epithelial cells, possibly because low titers of antibodies against epitopes of both LPS and 40-kDa OMP were generated in sera of mice immunized with WCV. Similarly, low titers of these antibodies against LPS and 40-kDa OMP may be present in humans vaccinated with WCV, which could explain why immunization with this vaccine failed to protect against *B. parapertussis* (7, 10, 19, 27).

Together, our data imply that the present pertussis vaccines may not be effective against *B. parapertussis*. However, cross-protection can be improved by incorporating surface molecules such as the 40-kDa OMP in these vaccines.

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