Bordetella pertussis attachment to respiratory epithelial cells can be impaired by fimbriae-specific antibodies

María Eugenia Rodríguez[1,2], Sandra M.M. Hellwig[2,4]*, María L.A. Pérez Vidakovics[1], Guy A.M. Berbers[4] & Jan G. J. van de Winkel[2,3]

[1]CINDEFI, Faculty of Science, La Plata University, La Plata, Argentina; [2]Immunotherapy Laboratory, Department of Immunology; [3]Genmab, University Medical Center, Utrecht, The Netherlands; and [4]Laboratory for Vaccine Preventable Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

Abstract

Bordetella pertussis attachment to host cells is a crucial step in colonization. In this study, we investigated the specificity of antibodies, induced either by vaccination or infection, capable of reducing bacterial adherence to respiratory epithelial cells. Both sera and purified anti-*B. pertussis* IgG or IgA fractions efficiently reduced attachment. This effect was found to be mediated mainly by fimbriae-specific antibodies. Antibodies with other specificities did not significantly interfere in the interaction of *B. pertussis* with respiratory epithelial cells, with the exception of antifilamentous hemaglutinin antibodies, which reduced bacterial attachment. However, this effect was smaller in magnitude than that observed in the presence of fimbriae-specific antibodies. The strong agglutinating activity of antifimbriae antibodies seems to be involved in this phenomenon.

Introduction

Bordetella pertussis is the etiologic agent of whooping cough, a disease that is re-emerging in many parts of the world despite high vaccination coverage. The side effects of whole-cell pertussis vaccines have prompted the development of acellular vaccines containing purified *B. pertussis* virulence factors. However, the combination of antigens inducing optimal efficacy is still unclear. Detailed insight into mechanisms protective against *B. pertussis* should facilitate a more rational selection of vaccine components.

Bordetella pertussis is an obligate human pathogen infecting the respiratory tract. The interaction between *B. pertussis* and the host involves several virulence factors. Surface-associated proteins such as fimbriae (Fim), filamentous hemaglutinin (FHA) and pertactin (Prn), have all been implicated in bacterial attachment to host cells, whereas toxins such as pertussis toxin (Ptx), adenylate cyclase toxin (Cya), lipopolysaccharide (LPS), among others, are regarded as being responsible for pertussis symptoms (Hewlett, 1997; Locht, 1999).

The relative contribution of humoral and cellular immunity on host protection against *B. pertussis* is still under investigation. However, efficient bacterial elimination from the respiratory tract might require both. Antibodies against several virulence factors were found to be correlated with clinical protection in humans (Cherry *et al.*, 1998; Storsaeter *et al.*, 1998). Antibodies may provide protection in several ways. They may neutralize toxins, interfere with attachment to respiratory cells, or facilitate bactericidal activity by immune cells. We have previously documented that *B. pertussis*-specific antibodies are required for the induction of cellular bactericidal effector functions (Rodríguez *et al.*, 2001a), and that Prn-specific antibodies play a key role in promoting *B. pertussis* uptake by professional phagocytes (Hellwig *et al.*, 2003). The specificity of antibodies capable of preventing attachment, however, remains unclear. Studies have shown the existence of antiadherence activity in sera from individuals either vaccinated or infected by *B. pertussis* (Tuomanen *et al.*, 1984). The authors detected this activity both in IgG and IgA antibody classes, but they did not

investigate the specificity of the antibodies involved. Both anti-FHA and anti-PT mouse antibodies have been shown to be capable of decreasing *B. pertussis* attachment to mouse fibroblasts (Lenin *et al.*, 1986). Later studies demonstrated that mouse antibodies with other specificities are able to reduce *B. pertussis* attachment to bronchial epithelial cells (van den Berg *et al.*, 1999).

The present study was performed to investigate further the specificity of antibodies, induced by vaccination or infection, that efficiently prevent bacterial interaction with the respiratory epithelia.

Material and methods

Bacterial strains and growth conditions

The virulent *B. pertussis* strain B213, a streptomycin-resistant derivative of Tohama, BpGR4, a Tohama-derived mutant lacking expression of FHA (Locht *et al.*, 1992), and strain B316, a Tohama-derived mutant lacking expression of Fim2, Fim3 and FimD (Geuijen *et al.*, 1996), were used in this study.

Bacteria were stored at $-70\,^{\circ}$C and recovered by growth on Bordet–Gengou (BG) agar plates at 35 $^{\circ}$C for 3 days. Virulent bacteria were subsequently plated on BG plates, cultured overnight, and used in the experiments.

Sera and antibodies

Sera from 24 Dutch children enrolled in a clinical trial to study efficacy of pertussis vaccines (Berbers *et al.*, 1999) were used in this study (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. Children were vaccinated with Dutch whole-cell vaccine in the first year of life and boostered with: (1) an acellular pertussis vaccine

Table 1. Specific antibody titers and agglutinating activity of serum samples from donors after pertussis booster vaccination (serum 1–24), and IgG and IgA fractions of pooled sera from pertussis-infected donors

| Donor | Virulence factors[*][†] | | | | Agglutinating activity | Attachment level[‡][§] (bacteria per cell) |
	PTx	FHA	Prn	Fim		
1	16	607	828	96	32	150
2	68	222	349	64	32	120
3	170	1332	599	175	64	180
4	1065	2232	454	457	256	74
5	148	981	305	76	32	159
6	30	199	33	12	4	241
7	1411	430	156	212	256	92
8	24	48	518	45	16	193
9	81	66	26	43	32	233
10	109	2148	156	136	128	112
11	83	138	120	69	8	192
12	69	1293	154	23	4	243
13	342	279	339	157	8	202
14	8	107	78	57	4	223
15	496	109	517	158	64	120
16	98	232	56	94	64	138
17	1	45	56	1478	512	5
18	6	33	115	152	128	105
19	52	100	104	344	256	61
20	6	31	7	174	256	72
21	105	79	44	416	256	52
22	25	28	32	149	128	120
23	36	24	25	855	1280	53
24	3	109	333	281	512	51
IgG	4354	1247	822	2487	800	8
IgA	93	310	73	954	400	18

[*]Antibody titers are expressed in ELISA units per mL calculated relative to the US reference pertussis antiserum.
[†]Preimmune sera showed no detectable antibody titers against *Bordetella pertussis*.
[‡]By means of four independent determinations.
[§]No significant differences of bacterial attachment levels were detected in the presence or the absence of the different preimmune sera.
Ptx, pertussis toxin; FHA, filamentous hemaglutinin; Prn, pertactin.

consisting of Ptx, FHA and Prn (GSK Rixensart Belgium, donors 1–14); (2) an acellular pertussis vaccine consisting of Ptx, FHA, Prn and Fim (Wyeth Lederle/Takeda, donors 15 and 16) or (3) Dutch whole-cell vaccine (donors 17–24) at 4 years of age. Sera samples were taken before and after vaccination. IgG and IgA fractions from pooled sera of pertussis-infected individuals were purified as described (van der Pol *et al.*, 2000). Depletion of either anti-Fim or anti-Prn antibodies from sera, and IgG- or IgA-containing fractions was performed as in Hellwig *et al.* (2003). The purity of both Fim2,3 and Prn antigens used for depletion was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analyses. Incubation of sera with the different proteins (Fim2,3 or Prn), selectively depleted antibodies specific for the respective antigen. The titers of antibodies with the other specificities (namely, FHA, PTx, and either Prn or Fim) remained unchanged, as assessed by enzyme linked immunosorbent assay (ELISA) before and after depletion. In order to rule out a possible role of the presence of purified Prn or Fim on bacterial adherence, both proteins were tested for their ability to influence bacterial attachment in the concentrations used for depletion. No effect on bacterial adherence to epithelial cells could be detected in the presence of any of these proteins (data not shown).

Rabbit anti-Fim serum was obtained as follows. Pathogen-free rabbits were immunized with $500 \mu g$ of purified Fim2,3 (Netherlands Vaccine Institute, Bilthoven, The Netherlands). Further immunizations were performed at 3 and 6 weeks. Sera were collected 7 weeks after primary immunization.

The following monoclonal antibodies (mAbs) against *B. pertussis* antigens were used: 118E10 (IgG1) against *B. pertussis* Fim, 13E11 (IgM) against *B. pertussis* Prn, 4-37F3 (IgG1) against *B. pertussis* FHA (Poolman *et al.*, 1990) (all kindly provided by the Netherlands Vaccine Institute, National Institute of Public Health and the Environment, The Netherlands).

Cells and growth conditions

The human alveolar epithelial cell line A549 (ATCC CCL185) (Rockville, MD, USA) was grown in RPMI 1640 medium (Gibco, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U mL^{-1} penicillin, and $100 \mu g \text{ mL}^{-1}$ streptomycin (Sigma, St Louis, MO, USA) (complete medium) at 37 °C in 5% CO_2.

ELISA and bacterial agglutination

Antibody titers against Ptx, FHA, Prn, and Fim2 were determined by ELISA as described previously (Meade *et al.*, 1995). Briefly, 96-well plates were coated with purified Ptx,

FHA, Fim2 (all from the Netherlands Vaccine Institute), or Prn (from Chiron, Siena, Italy) at concentrations of 1, 2, and $3 \mu g \text{ mL}^{-1}$, respectively. For each serum, a series of eight two-fold dilutions was tested, starting with a 1 : 60 dilution. Bound IgG was detected after 2 h incubation (room temperature) with alkaline phosphatase-conjugated goat antihuman IgG (Sigma) (working dilution: 30 000). ELISA units (EU) were calculated relative to the US Reference Pertussis Antiserum (human, obtained from the FDA), lot 3 for Ptx, FHA and Fim, and lot 4 for Prn using the four parameter fit method in KC4 (Kineticalc for Windows), with a BioTek plate reader (EL312e, BioTek, Winooski, VT, USA). The minimum level of detection was estimated at 2 EU mL^{-1} for anti-Ptx and anti-FHA antibodies, 4 EU mL^{-1} for anti-Prn antibodies, and 2 EU mL^{-1} for anti-Fim2 antibodies.

Agglutinating antibodies were measured essentially as described by Nagel *et al.* (1985) using *B. pertussis* strain 3838 as antigen. US Pertussis antiserum was used as a reference and a control serum was included on each plate. For each serum, a two-fold dilution series in eight wells was tested (starting with a 1 : 4 dilution). The agglutination titer is expressed as the reciprocal of the highest final dilution of serum inducing agglutination.

Attachment assays

Attachment assays were performed as described previously (Sisti *et al.*, 2002) with minor modifications. Briefly, A549 cells were grown in complete medium using 24-well tissue culture plates (Nunc, Roskilde, Denmark) for 18 h. Monolayers were washed twice with RPMI 1640 medium supplemented with 10% FCS prior to incubation with virulent *B. pertussis* (500 bacteria per cell) in the absence or presence of sera (0–10% v/v), purified IgG (0–100 $\mu g \text{ mL}^{-1}$), purified IgA (0–100 $\mu g \text{ mL}^{-1}$), or mAbs (0–30 $\mu g \text{ mL}^{-1}$). Bacterial inocula were quantified using appropriate dilutions onto BG plates. To facilitate bacterial interaction with epithelial cells, plates were centrifuged for 5 min at 640 *g*. Adherence of bacteria to A549 cells was determined after 2 h of incubation at 37 °C (with 5% CO_2). No changes in the number of viable A549 cells were detected upon infection. Experiments were stopped by washing the monolayers with RPMI 1640 medium supplemented with 10% FCS, prior to fixation with methanol. Bacterial adherence was quantified by microscopy after crystal violet staining. Adherent bacteria were counted by microscopic examination of 20 randomly-selected fields, showing a minimum of seven epithelial cells per field.

Phagocytosis

Phagocytosis of opsonized *B. pertussis* was performed as described in Rodríguez *et al.* (2001a, b) with minor

modifications. Briefly, GFP-labeled *B. pertussis* were incubated with 10% (v/v) serum, IgG (300 μg mL^{-1}), or IgA (300 μg mL^{-1}), for 30 min at 37 °C. Opsonized bacteria were allowed to attach to freshly isolated human polymorphonuclear leucocyte cells in a ratio of 70 : 1 for 30 min at 4 °C. After extensive washing to remove nonattached bacteria, cells were split into two aliquots, and further incubated for 30 min at either 4 or 37 °C. Subsequently, remaining cell surface-bound opsonized bacteria were detected by incubation for 30 min at 4 °C with phycoerythrin (PE)-conjugated goat F(ab′)$_2$ fragments of antihuman IgG, or PE-conjugated goat F(ab′)$_2$ fragments of antihuman IgA (Southern Biotechnology, Birmingham, AL, USA). After washing, samples were analyzed using flow cytometry. Five thousand cells were analyzed per sample. Green and red fluorescence intensities of cells maintained at 4 °C served as control for bacterial binding (i.e. 0% phagocytosis). The decrease in red fluorescence of green cells after incubation at 37 °C reflected bacterial phagocytosis, and were used to calculate phagocytosis as described previously (Rodriguez *et al.*, 2001b).

Statistical analyses

Antibody titers were converted to log 10 values before analyses for linear correlation. Pearson's product moment correlation coefficient was calculated for the inverse of log-converted antibody titers against selected bacterial antigens and bacterial attachment levels. Student's *t*-tests were used to assess the significance of differences between averages. Significance was accepted at $P < 0.05$.

Results

The A549 cell line has previously been used to investigate the involvement of *B. pertussis* virulence factors on bacterial interaction with human respiratory epithelial cells (Schipper *et al.*, 1994; Alonso *et al.*, 2001; Ishibashi *et al.*, 2001; Coutte *et al.*, 2003). We employed this model system to study the capacity of specific antibodies to prevent *B. pertussis* attachment to respiratory cells. As reported before by Tuomanen *et al.* (1984), using human ciliated tracheal cells to study *B. pertussis* attachment, we observed that the addition of sera from pertussis-vaccinated individuals with detectable titers of specific antibodies decreased bacterial adherence to A549 cells (Table 1). Regression analyses ($n = 24$) suggested that this decrease was mainly due to the presence of antibodies directed against Fim ($r = 0.84$, $P < 0.05$). The level of antibodies against Prn, Ptx, or FHA showed no correlation with bacterial attachment decrease ($r = -0.125$, $P < 0.05$; $r = -0.058$, $P < 0.05$ and $r = -0.30$, $P < 0.05$, respectively). Serum samples obtained prior to booster vaccination, without detectable antibody titers against *B. pertussis*, did

not influence bacterial attachment to respiratory epithelial cells (Fig. 1a).

To investigate the importance of Fim-specific antibodies, further sera with different levels of anti-Fim antibodies (serum 2 and serum 4, Table 1) were depleted of antibodies aganist Fim or Prn (the latter used as a control). As observed before (Hellwig *et al.*, 2003), antibodies directed against the other *B. pertussis* antigens tested (namely, FHA, Ptx, and Prn) remained unaffected by this procedure as checked by ELISA (data not shown). In agreement with the regression analyses, depletion of anti-Fim antibodies abrogated almost completely the inhibitory effect of anti-*B. pertussis* antibodies on bacterial attachment, whereas depletion of Prn antibodies did not have an effect (Fig. 1a).

Similar results were obtained with *B. pertussis* IgA and IgG purified from sera of pertussis patients with high titers against *B. pertussis*. Both isotypes proved to be capable of interfering with bacterial attachment (Table 1) and Fim-directed antibodies were shown to play the main role. Depletion of anti-Fim, but not anti-Prn IgG or IgA, abrogated the inhibition of bacterial attachment (Fig. 1a).

This activity of anti-Fim antibodies was further confirmed using two different sources of antibodies, a rabbit anti-Fim serum and anti-Fim monoclonal antibodies (mAbs). Both anti-Fim rabbit antiserum and mAbs efficiently reduced bacterial attachment (Fig. 1b), whereas anti-Prn mAb did not (Fig. 1b). Figure 1c shows that bacterial attachment inhibition with anti-Fim antibodies is dose-dependent, whereas no influence of anti-Prn antibodies on bacterial adherence was detected using equivalent concentrations. Similar results were obtained using Fim and Prn antisera (data not shown).

Importantly, FHA but not Fim has been described previously as the main adhesin of *B. pertussis*. However, antibodies against FHA proved to be capable of reducing bacterial attachment less efficiently ($P < 0.05$) than anti-Fim antibodies (Fig. 1d). We investigated therefore the relative importance of these two adhesins on *B. pertussis* attachment to the human cells used in our study (A549). Using *B. pertussis* mutants lacking expression of either FHA (BpGR4) or both the major and the minor Fim subunits (Bp316), we found FHA but not Fim to be crucial for *B. pertussis* attachment to A549 cells (Fig. 1d).

Together, these results suggest that anti-Fim antibodies do not primarily block adhesive epitopes on the fimbrial protein, but they interfere with bacterial attachment by a different Fim-dependent mechanism. In agreement with this hypothesis, we found that, although the *B. pertussis* isogenic mutant lacking expression of Fim proved to adhere to epithelial cells in a similar way as did wild-type bacteria, the attachment of Fim-mutant isogenic strain was not inhibited by immune sera that efficiently decreased the wild-type *B. pertussis* (B213) attachment level (Fig 1d).
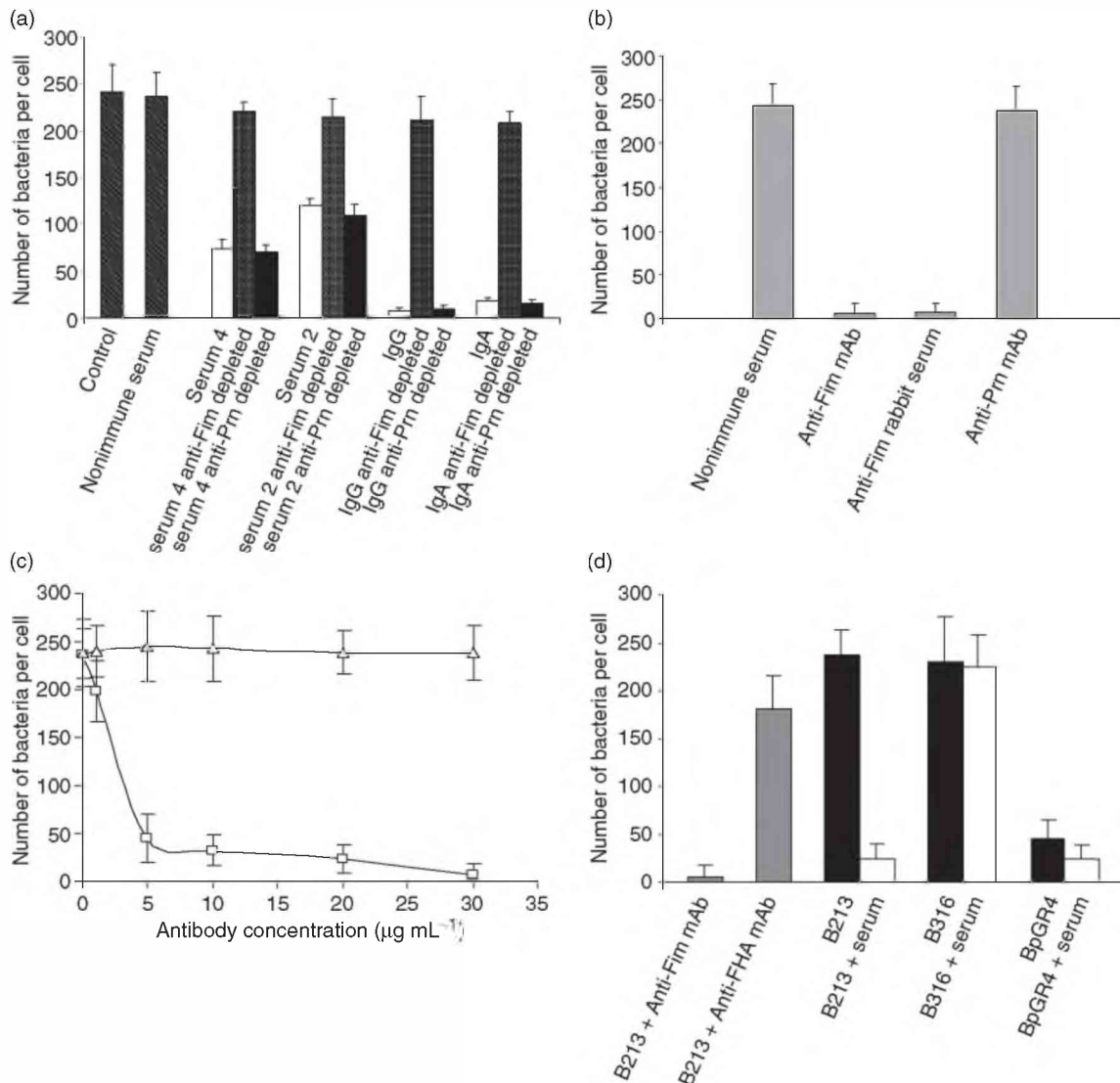
Fig. 1. Effect of specific antibodies on *Bordetella pertussis* attachment to human respiratory epithelial cells, A549. (a) *B. pertussis* was added to A549 cell monolayers in the presence or absence of serum, purified IgG, and IgA isotypes (10% v/v, 100 and 100 μg mL^{-1}, respectively) before (serum, IgG, IgA), and after depletion of either Fim-directed antibodies (serum anti-Fim depleted, IgG anti-Fim depleted, IgA anti-Fim depleted) or Prn directed antibodies (serum anti-Prn depleted, IgG anti-Prn depleted, IgA anti-Prn depleted). Bacterial attachment to epithelial cells in the absence of antibodies (control) or in the presence of serum with no detectable antibodies to *B. pertussis* (nonimmune serum, 10% v/v) was determined in every assay. Data represent mean $\pm$ SD of four independent experiments. (b) Anti-Fim and anti-Prn monoclonal antibodies (30 μg mL^{-1}), and rabbit anti-Fim antibodies (30 μg mL^{-1}) were tested for their ability to reduce *B. pertussis* attachment to epithelial cells. Nonimmune serum was included in every experiment as a control. Data represent mean $\pm$ SD of four independent experiments. (c) Effect of anti-Fim ($\square$), and anti-Prn ($\triangle$) monoclonal antibody concentrations on *B. pertussis* attachment to A549 cells. The different concentrations were tested in three independent experiments. The decrease of bacterial attachment level proved significant in the presence of 5, 10, 20 and 30 μg mL^{-1} anti-Fim antibodies. Data represent mean $\pm$ SD. (d) Relevance of Fim and FHA as adhesins and/or targets for antibody-dependent *B. pertussis* attachment inhibition. Attachment of *B. pertussis* (B213), *B. pertussis* Fim deficient mutant (B316), and *B. pertussis* FHA deficient mutant (BpGR4) to epithelial cells in the presence and absence of immune serum (serum 4, 10% v/v) was determined. Attachment of *B. pertussis* (B213) to respiratory cells was further evaluated in the presence of either anti-Fim (30 μg mL^{-1}) or anti-FHA (30 μg mL^{-1}) antibodies. Data represent mean $\pm$ SD of four independent experiments.

Anti-Fim antibodies have previously been described as agglutinins (Li *et al.*, 1988a). Bacterial agglutination may preclude attachment. Similarly, we found sera agglutinating activity highly correlated with both anti-Fim antibody titers ($r = 0.81$, $P < 0.05$) and the decrease in attachment levels of wild-type bacteria ($r = 0.82$, $P < 0.05$). Microscopic evaluation of *B. pertussis* incubated with either immune sera (containing high titers of anti-Fim antibodies) or rabbit
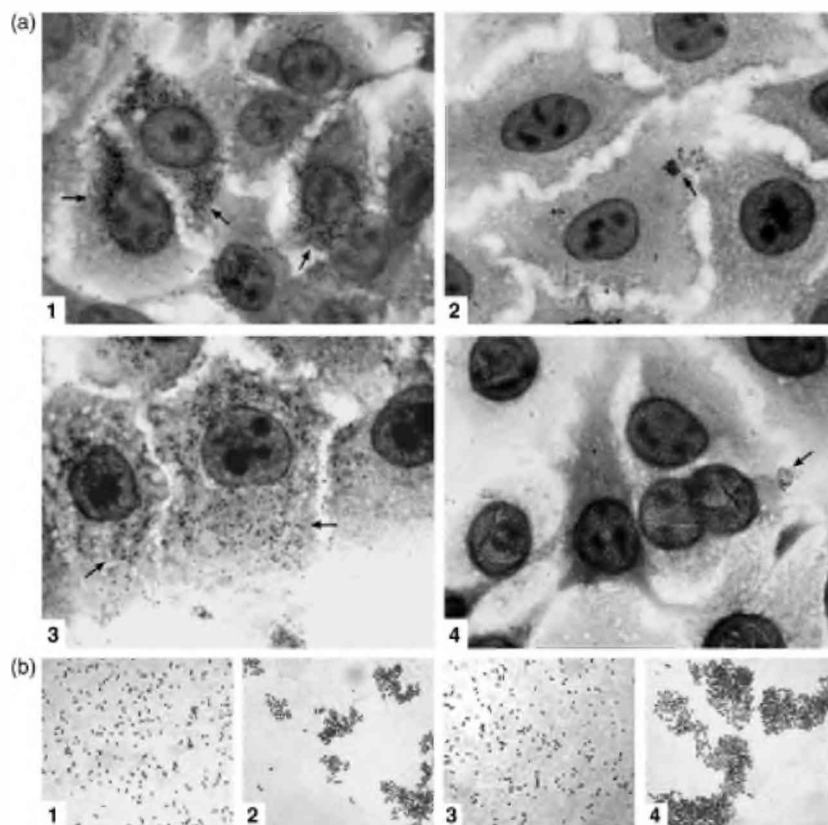
Fig. 2. Anti-Fim antibodies and *Bordetella pertussis* attachment to A549 cells. (a) *B. pertussis* were incubated for 2 h with A549 cells in the absence (1), or presence of immune serum (2), immune serum depleted of anti-Fim antibodies (3), or rabbit *B. pertussis* anti-Fim antibodies (4). Bacteria can be observed in association with A549 cells (solid arrows). (b) Agglutinating activity of anti-Fim antibodies. *Bordetella pertussis* were incubated for 30 min at 37 °C with (1) medium alone, RPMI 1640 medium supplemented with 10% FCS, (2) immune serum, (3) immune serum depleted of anti-Fim antibodies, or (4) rabbit *B. pertussis* anti-Fim antibodies. Panels are representative of one out of five independent experiments.

anti-Fim serum, showed wild-type bacteria highly agglutinated, but not the Fim-deficient mutant. Figure 2 shows that serum depletion of Fim-directed antibodies determined the loss of agglutinating activity and an increase in the number of attached bacteria per cell to a level close to that achieved in the absence of serum. Additionally, although neither Fim- nor Prn-directed monoclonal antibodies agglutinated bacteria when tested at $1 \mu g \, mL^{-1}$, monoclonal antibodies directed against Fim, but not against Prn, proved capable of agglutinating wild type bacteria when tested at 5, 10, 20 and $30 \mu g \, mL^{-1}$ (data not shown). These were concentrations at which bacterial attachment was found to be drastically decreased (see Fig. 1c).

Antibody-mediated phagocytosis plays a central role in the induction of bactericidal cellular effector functions (Rodríguez *et al.*, 2001a). Agglutination of bacteria may facilitate bacterial uptake by professional phagocytes. We assessed therefore the opsonic activity of sera that showed high agglutinating activity before and after anti-Fim antibody depletion. The lack of agglutinating activity after anti-Fim antibody depletion was checked by microscopy. Sera from children that had been vaccinated, and purified IgG and IgA from infected individuals, induced efficient phagocytosis of *B. pertussis* (Fig. 3a). However, Fim-specific

antibodies and bacterial agglutination were not found to be relevant for bacterial uptake by neutrophils (Fig. 3b).

Discussion

Bordetella pertussis is an obligate human pathogen that resides in the respiratory tract during infection. Bacterial interaction with human respiratory epithelial cells theoretically constitutes a key event in host colonization and whooping cough pathogenesis. Host cell surface receptors and several bacterial factors are thought to be involved in adhesion of *B. pertussis*, and a number of adhesins have been identified (Leininger *et al.*, 1992; Kerr & Matthews, 2000). Despite a large number of studies dealing with bacterial factors involved in this process, there are few data on the capacity of specific antibodies to prevent *B. pertussis* attachment to host cells. Previous work has shown that the presence of immune sera decreases *B. pertussis* attachment to human ciliated cells. This antiadherence activity was reported for both IgG and IgA fractions (Tuomanen *et al.*, 1984), yet the specificity of the antibodies involved was not identified. Consistent with these results, we found sera from pertussis-vaccinated individuals that efficiently decreased *B. pertussis* attachment to respiratory cells. Preimmune sera,
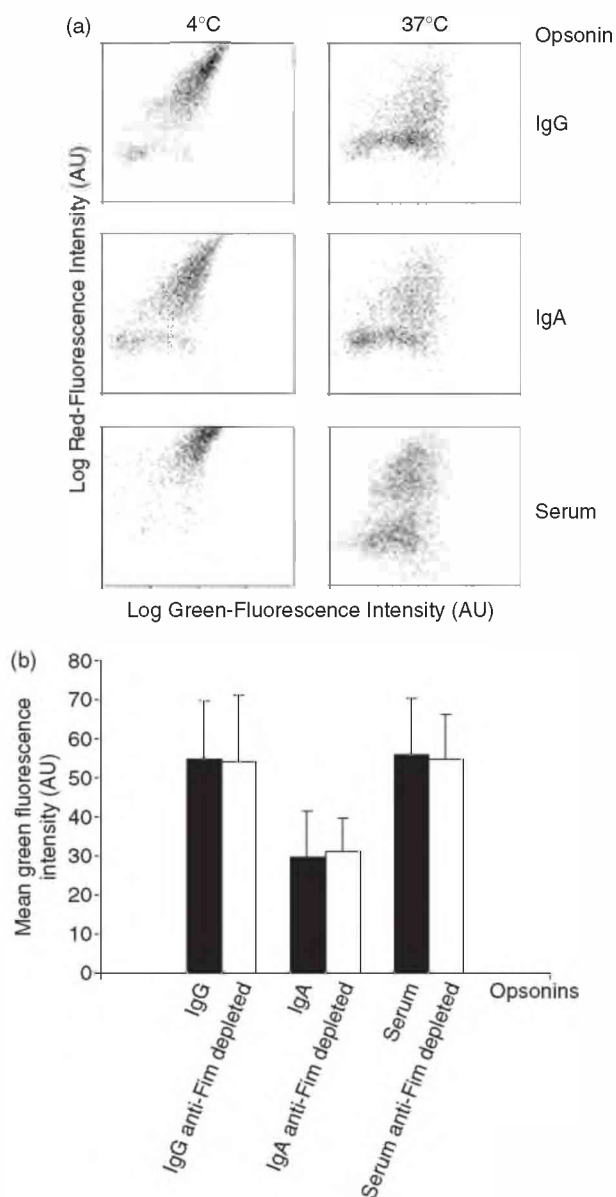
Fig. 3. Effect of Fim-directed antibodies on *Bordetella pertussis* phagocytosis by human PMN. (a) Serum, IgG and IgA isotypes were tested in their ability to induce phagocytosis of *B. pertussis*. Opsonized GFP-expressing *B. pertussis* were incubated with PMN at 4 °C for 30 min. Suspensions were then split in two aliquots, and subsequently incubated for 30 min at either 4 or 37 °C. Remaining surface-bound IgG-opsonized *B. pertussis* were detected by addition of PE-conjugated goat F(ab')$_2$ fragments of antihuman IgG antibodies. (b) PMN phagocytosis of *B. pertussis* opsonized with serum, IgG, and IgA before (serum, IgG, IgA), and after anti-Fim antibody depletion (serum anti-Fim depleted, IgG anti-Fim depleted, IgA anti-Fim depleted). Data represent mean ± SD of five independent experiments, performed with PMN isolated from different donors.

lacking detectable antibody titers against pertussis, exhibited no influence on bacterial adherence levels, confirming the importance of specific antibodies on attachment inhibition.

The analysis of 24 sera samples taken after pertussis vaccination showed that Fim-specific antibodies play a major role in such inhibition. We further found both IgA and IgG isotypes to be responsible for reducing *B. pertussis* attachment. In the case of serum, this activity proved to be primarily dependent on the level of Fim antibodies present in those fractions. However, previous studies performed with A549 or other cell lines (Relman *et al.*, 1989; van den Akker, 1998; Alonso *et al.*, 2001; Coutte *et al.*, 2003) had shown FHA but not Fim to be the main adhesin of *B. pertussis*. Accordingly, we observed that the lack of expression of FHA determined a drastic decrease on bacterial attachment to A549 cells, whereas the attachment efficiency of both wild type *B. pertussis* and *B. pertussis* mutant lacking expression of fimbriae were equivalent. FHA directed antibodies, however, were shown to have a moderate effect on *B. pertussis* attachment compared to the decrease observed in the presence of Fim-directed antibodies. These results indicate that *B. pertussis* fimbriae are not critical for bacterial attachment, but rather constitute a target for efficient antibody-mediated prevention of bacteria–epithelial cell interaction. Consistent with this hypothesis, the presence of sera capable of blocking wild-type *B. pertussis* attachment did not modify Fim-deficient isogenic strain adherence. Antibody-mediated bacterial agglutination seems to underlie this phenomenon. Fim-directed antibodies and antibodies aganist Prn and LPS had been previously described as agglutinins (Li *et al.*, 1988a, b; Brennan *et al.*, 1988). However, as found by others (Mink *et al.*, 1994), our results showed anti-Fim antibodies to be the main agglutinin present in both postvaccination and postinfection human sera. Human sera containing anti-Fim antibodies, but not sera depleted of anti-Fim antibodies, induced wild-type bacteria agglutination. Additionally, human sera capable of agglutinating wild-type *B. pertussis* strain failed to agglutinate Fim-deficient isogenic strain at the same concentrations. Microscopic analysis further confirmed that only under agglutinating conditions is there a drastic reduction in the number of bacteria attached to the respiratory cell.

These results support the hypothesis that the ability of anti-Fim antibodies to reduce bacterial attachment might primarily be related to the induction of bacterial agglutination. The strong correlation between the decrease in bacterial attachment levels and both the sera agglutinating activity and the Fim antibody level is consistent with this hypothesis. Previous results reported by van den Berg *et al.* (1999) suggested that antibodies directed against any surface structure of *B. pertussis* would interfere with bacterial adherence to bronchial epithelial cells. Having performed the studies using nonagglutinating concentrations of each antibody tested, as reported in their article, the moderate effect of anti-Fim antibodies on *B. pertussis* attachment seems to be consistent with our findings.

Sera agglutinating activity have been found correlated with protection in early vaccine trials (Medical Research Council, 1956). The presence of this activity in human sera was later attributed mainly to Fim antibodies (Mink *et al.*, 1994), but the mechanism by which agglutinins provide protection was not elucidated. Bacterial aggregation, previously found to be detrimental for colonization (Coutte *et al.*, 2003), may preclude efficient bacteria–cell interaction, but may also facilitate bacterial clearance by immune cells. In this study we found bacterial agglutination not to influence *B. pertussis* phagocytosis significantly, but rather to interfere with bacterial attachment to host cells. The data stress the importance of multivalency of vaccines. Such vaccines are not only capable of inducing high titers, but may also facilitate the induction of multiple and synergistic lines of host defense.

In summary, our data suggest that anti-Fim antibodies induced by either vaccination or infection to promote a specific mechanism of host defense by interfering *Bordetella*–respiratory cell interaction. These results provide a biological basis for the field trial investigation, reporting increased protection against *B. pertussis* by vaccines including Fim, and the reported association of high antibody titers against Fim with a lower likelihood of acquiring pertussis (Cherry *et al.*, 1998; Storsaeter *et al.*, 1998).

Acknowledgments

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