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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Received 24 September 1998/Returned for modification 9 November 1998/Accepted 10 December 1998

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, diagnosis of 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), 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*, FHA, PTg, Prn, and fimbriae, or against two other surface molecules, lipopolysaccharide (LPS) and the 40-kDa outer membrane porin protein (OMP), 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.

**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 (Difeo 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 10^8 CFU/ml in HAP medium (PBS containing 3 mM glucose, 150 mM CaCl_2, 500 mM MgCl_2, 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); purified FHA and Prn and genetically detoxified PT (PTg) were kindly provided by R. Rappuoli (Biocine SpA, Siena, United Kingdom).
immunoglobulin G (IgG), IgA, or IgM (Southern Biotechnology Associates Inc. Netherlands) and H2O2 (0.003%) in 110 mM sodium acetate buffer (pH 5.5) was coated overnight with 5 μg of goat anti-mouse IgG, IgA, or IgM (Cappel Research Products, Durham, N.C.) per ml in 50 mM sodium carbonate buffer (pH 9.6) at 3°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 purified IgG, IgA, or IgM (SBA) as the standard.

**MAbs.** The following monoclonal antibodies (MAbs) against B. pertussis surface proteins were used as ascites fluid: 4-373 (IgG1; 6.8 mg/ml) against B. pertussis FHA (31), 36G3 (IgG1, 1.9 mg/ml) against B. pertussis lipopolysaccharide (LPS) (31), and 30E5 (IgG2b, 14.5 mg/ml) against B. pertussis 40-kDa outer membrane porin protein (OMP) (31) (all kindly provided by J. Poolman, National Institute of Public Health and the Environment, The Netherlands). The MAbs were used in a final concentration of 2 μg/ml.

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 × 10^6 heat-killed B. pertussis or B. parapertussis per ml suspended in a 50 mM sodium carbonate buffer (pH 9.6) at 3°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}. Concentrations of immunoglobulin subclasses of the antibodies in the mouse sera were determined by ELISA as described above except that the plates were coated overnight with 5 μg of goat anti-mouse IgG, IgA, or IgM and incubated for 1 h at 3°C. After five washes with PBS at room temperature, the plates were examined with fluorescence microscopy at a magnification of ×400. 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 3°C for 4 h. After 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 WCV**

Sera of mice immunized with purified B. pertussis virulence factors FHA, PTg, fimbriae, and Prn 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

### 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 concen (μg/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgA</td>
</tr>
<tr>
<td>Normal mouse serum</td>
<td>ND</td>
<td>196 ± 14</td>
</tr>
<tr>
<td>Serum of mice immunized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFA</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>Fimbriac</td>
<td>6.0 ± 0.89</td>
<td>3,162 ± 382</td>
</tr>
<tr>
<td>Prn</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.
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, limbriae, 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 ± 13 (mean ± standard deviation [SD]) and 96 ± 29 bacteria/100 epithelial cells, respectively. Antiserum against **B. pertussis** FHA, PTg, limbriae, 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,

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**Table 2. Effects of antibodies against **B. pertussis** virulence factors on adherence of **B. pertussis** to epithelial cells**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Adherence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% of positive epithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No serum</td>
<td>96 ± 29</td>
<td>259 ± 33</td>
</tr>
<tr>
<td>Serum against:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>67 ± 21</td>
<td>264 ± 25</td>
</tr>
<tr>
<td>WCV</td>
<td>17 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>152 ± 34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FHA</td>
<td>11 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>135 ± 22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PTg</td>
<td>21 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>180 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fimbriae</td>
<td>14 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>168 ± 46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Prn</td>
<td>26 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>205 ± 9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined in the presence of 2.5\% control anti-TT serum, serum with antibodies against WCV, FHA, PTg, limbriae, or Prn, or HAP medium only; values are means ± SD of at least four separate experiments.

<sup>b</sup> \(P < 0.05\) versus anti-TT antibodies by ANOVA and Newman-Keuls multiple-comparison test.
the bacteria were preincubated with 1% antiserum against \textit{B. pertussis} FHA, PTg, fimbriae, Prn, WCV, or TT, or with HAP medium lacking serum, and next incubated with NCI-H292 cells. Preincubation of \textit{B. pertussis} with antiserum against the various \textit{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 \textit{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 \textit{B. pertussis} or \textit{B. parapertussis} to NCI-H292 cells in the presence of MAb against \textit{B. pertussis} FHA, LPS, or 40-kDa OMP.** Antibodies against the various virulence factors of \textit{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 \textit{B. pertussis} and \textit{B. parapertussis} (2, 3, 8, 11, 29), but these surface antigens are not implicated in the adherence of \textit{B. pertussis} to respiratory epithelial cells (39). Using an ELISA technique, we found that both \textit{B. pertussis} and \textit{B. parapertussis} bound MAb against LPS or 40-kDa OMP, whereas \textit{B. pertussis} but not \textit{B. parapertussis} bound MAb against FHA (Table 4).

**Adherence of \textit{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 \textit{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 \textit{B. pertussis} virulence factors FHA, PTg, fimbriae, and LPS bound MAb against LPS or 40-kDa OMP, whereas \textit{B. pertussis} but not \textit{B. parapertussis} bound MAb against FHA (Table 4). Adherence of \textit{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 \textit{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).

**TABLE 3. Effects of preincubation of \textit{B. pertussis} and \textit{B. parapertussis} with antisera against various \textit{B. pertussis} virulence factors or WCV on the adherence to bronchial epithelial cells.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>\textit{B. pertussis}</th>
<th>\textit{B. parapertussis}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of bacteria/100 cells</td>
<td>% inhibition</td>
</tr>
<tr>
<td>No serum</td>
<td>118 ± 24</td>
<td>77 ± 30</td>
</tr>
<tr>
<td>Serum against:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>118 ± 15</td>
<td>83 ± 31</td>
</tr>
<tr>
<td>FHA</td>
<td>44 ± 17(^{b})</td>
<td>74 ± 16</td>
</tr>
<tr>
<td>PTg</td>
<td>64 ± 8(^{c})</td>
<td>70 ± 18</td>
</tr>
<tr>
<td>Fimbriae</td>
<td>43 ± 14(^{c})</td>
<td>84 ± 23</td>
</tr>
<tr>
<td>Prn</td>
<td>57 ± 26(^{c})</td>
<td>75 ± 21</td>
</tr>
<tr>
<td>WCV</td>
<td>75 ± 15(^{c})</td>
<td>70 ± 5</td>
</tr>
</tbody>
</table>

\(^{a}\) Bacteria were preincubated with 1% antiserum, after which adherence was determined. Results of a duplicate representative experiment are shown.

\(^{b}\) Compared to adherence in the presence of anti-TT antibodies.

\(^{c}\) \(P < 0.05\) versus anti-TT antibodies (control) by ANOVA and Newman-Keuls multiple-comparison test.

**TABLE 4. Antibody responses of MABs against \textit{B. pertussis} surface antigens to \textit{B. pertussis} and \textit{B. parapertussis}**

<table>
<thead>
<tr>
<th>MAb against:</th>
<th>Clone</th>
<th>Isotype</th>
<th>Antibody titre ((–\log_{10}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>\textit{B. pertussis}</td>
</tr>
<tr>
<td>FHA</td>
<td>37F3</td>
<td>IgG1</td>
<td>5.6</td>
</tr>
<tr>
<td>LPS</td>
<td>36G3</td>
<td>IgG1</td>
<td>5.8</td>
</tr>
<tr>
<td>40-kDa OMP</td>
<td>30E5</td>
<td>IgG2b</td>
<td>6.5</td>
</tr>
</tbody>
</table>

\(^{a}\) Bacteria were coated onto microtiter plates, and binding of the indicated MABs was determined as described in Materials and Methods.

\(^{b}\) Reciprocal of the highest dilution corresponding with three times the blank value. <LD, below the level of detection.
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, Prn, and 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, antisera 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 antisera 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, antisera 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.

ACKNOWLEDGMENTS

We express our gratitude to Rob Willems for helpful discussions. This work was financially supported by Preaventi Fonds grant 2825450.

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Editor: D. L. Burns