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Bordetella pertussis and *Bordetella parapertussis*: Two Immunologically Distinct Species

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***Bordetella pertussis* and *Bordetella parapertussis* are closely related species. Both are responsible for outbreaks of whooping cough in humans and produce similar virulence factors, with the exception of pertussis toxin, specific to *B. pertussis*. Current pertussis whole-cell vaccine will soon be replaced by acellular vaccines containing major adhesins (filamentous hemagglutinin and pertactin) and major toxin (pertussis toxin). All of these factors are antigens that stimulate a protective immune response in the murine respiratory model and in clinical assays. In the present study, we examined the protective efficacies of these factors, and that of adenylate cyclase-hemolysin, another *B. pertussis* toxin, against *B. parapertussis* infection in a murine respiratory model. As expected, pertussis toxin did not protect against *B. parapertussis* infection, since this bacterium did not express this protein, but the surprising result was that none of the other factors were protective against *B. parapertussis* infection. Furthermore, *B. parapertussis* adenylate cyclase-hemolysin, although it protected against *B. parapertussis* infection, did not protect against *B. pertussis* infection. Despite a high degree of homology between both *B. pertussis* and *B. parapertussis* species, no cross-protection was observed. Our results outline the fact that, as in other gram-negative bacteria, *Bordetella* surface proteins vary immunologically.**

Whooping cough is an acute respiratory disease caused by *Bordetella pertussis*. This disease is characterized by paroxysmal cough with or without whoops, leukocytosis, hypoglycemia, histamine sensitivity, immobilization and/or damage of the cilia of the ciliated epithelium of the respiratory tract, alveolitis, and bronchopneumonia (28). *Bordetella parapertussis*, a closely related species, is also responsible for outbreaks of whooping cough in humans (20, 22, 28). That *B. parapertussis* and *B. pertussis* are closely related species has been well documented by DNA hybridization studies and isozyme comparisons (17, 26). Although both species produce a number of characterized virulence factors, including filamentous hemagglutinin (FHA) (6), fimbrial adhesin (25), pertactin (21), dermonecrotic toxin (14), tracheal cytotoxin (12), and adenylate cyclase-hemolysin (AC-Hly) (15), only *B. pertussis* produces the well-characterized pertussis toxin (PTX) (3). *B. parapertussis* carries the structural genes encoding PTX subunits, but these genes are not transcribed because of the presence of mutations in the promoter region (3, 23).

It is generally thought that *B. parapertussis* causes a mild whooping cough-like disease, whereas *B. pertussis* causes a severe one and that the nontoxinogenic nature of *B. parapertussis* infection is due to the fact that this bacterium does not express PTX. Monak et al. showed that expression of active PTX by *B. parapertussis* was correlated with the induction of leukocytosis, anaphylaxis, and histamine sensitivity (24). However, as emphasized by Novotny, "Both *B. pertussis* and *B. parapertussis* can induce mild or severe disease and no clinician in the world could distinguish between the two infections by clinical symptoms only" (28). Moreover, mild *B. parapertussis* infections may be a major cause of prolonged bronchitis. It is also thought that the frequency of *B. parapertussis* infections is low, but this could, in part, be due to the fact that identification of *B. parapertussis* is highly dependent on the experience of the laboratory isolating the bacteria and to the limited number of epidemiological studies addressing the issue of *B. parapertussis* infections. This frequency of infections may therefore be higher than anticipated. Although the degree of homology between the two species is very high, whole-cell *B. pertussis* vaccine and even pertussis infection do not seem to protect against *B. parapertussis* infection, as demonstrated by Lautrop and many other investigators (7, 13, 20, 27, 37).

Current acellular vaccines composed primarily of detoxified PTX alone are unlikely to protect against *B. parapertussis*-caused whooping cough because this antigen is not expressed by this bacterium. It may therefore be very important to include in acellular vaccine preparations other antigens known to be protective (i.e., to stimulate an effective immune response) against *B. pertussis* infection, such as FHA, pertactin, and AC-Hly (11, 16, 29, 31, 34), in order to protect against both *B. pertussis* and *B. parapertussis* infections.

In the present study, in order to document whether acellular vaccines may confer cross-protection against *B. parapertussis* infections, which current whole-cell vaccines do not seem to induce, we examined the protective efficacy of four purified *B. pertussis* antigens against *B. parapertussis* infections using a murine respiratory model. The results tend to show that cross-immunity between the two *Bordetella* species does not exist.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study were *B. pertussis* Tohama (CIP 8132), *B. pertussis* 18323, *B. parapertussis* (CIP 63.2), and *B. pertussis* Pillemer

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(P134) which was originally obtained from R. Preston (University of Glasgow, Glasgow, Scotland).

Bacteria were grown on Bordet Gengou agar supplemented with 15% defibrinated sheep blood (BG) at 36°C for 72 h and again for 24 h. Subculturing in liquid medium was performed in Stainer-Scholte medium (35) for 20 h at 36°C until the optical density at 650 nm reached 1.0. For Western blotting (immunoblotting) analysis, bacteria grown on BG were resuspended in saline at a concentration of 2×10^{10} CFU/ml, diluted in Laemmli buffer, and boiled for 15 min (19).

Protein purification. *B. pertussis* and *B. parapertussis* AC-Hly and AC fragments were purified from the bacteria or culture supernatant of *B. pertussis* Tohama and *B. parapertussis* 63.2, respectively, with a calmodulin affinity column, as described previously (16, 18). PTX and FHA were purified from the culture supernatant of *B. pertussis* Pillemer by the methods of Sekura et al. (33) and Sato et al. (30), respectively, with slight modifications. Briefly, FHA present in the concentrated supernatant was batch adsorbed to hydroxylapatite and the gel was packed into a column while the supernatant containing the toxin was further batch adsorbed on an asialofetuin-Sepharose 4B column. After different wash buffer cycles, the antigens were eluted with specific buffers. Pertactin was heat extracted from the bacterial pellet obtained after centrifugation, as described by Brennan et al. (9), and purified according to the procedure described by Burns et al., (10). Briefly, the extract was applied to a Sepharose 6B-DEAE column and the pertactin was eluted by an NaCl gradient. The fractions containing pertactin were further applied to an Affi-Gel blue column (Bio-Rad), and the protein was eluted with urea.

All preparations were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), tested in rabbits for pyrogen effect, and tested with an enzyme-linked immunosorbent assay and a CHO cell assay for cross-contaminations. AC-Hly, AC fragments, FHA, and pertactin were included in their native form in the vaccine preparations, while PTX was inactivated with glutaraldehyde to yield the pertussis toxoid. Protein concentrations were determined by the method of Bradford (8).

Electrophoresis and immunoblotting methods. SDS-PAGE was performed with ready-to-use 8 to 25% polyacrylamide gels and the Pharmacia PhastSystem. After electrophoresis, the proteins were transferred from polyacrylamide gels to Hybond C-Super membranes (Amersham). After blocking, membranes were incubated at a 10^{-3} dilution with polyclonal sera at 4°C overnight. The immunochemical detection was performed with horseradish peroxidase-labelled sheep anti-mouse immunoglobulins and an enhanced chemiluminescence system (Amersham).

Vaccine preparation. For acellular vaccines, glutaraldehyde pertussis toxoid, FHA, pertactin, AC-Hly, and AC fragments purified from *B. pertussis* and from *B. parapertussis* were adsorbed on aluminum hydroxide at a final aluminum concentration of 250 μg/ml.

Active immunizations. For active immunizations, female 3- to 4-week-old BALB/c mice (CERJ, St. Berthevin, France) were injected subcutaneously with 500 μl of each acellular vaccine twice with a 2-week interval between injections. Controls were injected with 250 μl of control buffer containing aluminum hydroxide. These doses corresponded, per injection, to 8 μg (each) of *B. pertussis* detoxified PTX, FHA, and pertactin; 4 μg of *B. pertussis* or *B. parapertussis* AC fragments; and 20 μg of *B. pertussis* AC-Hly. The mice were bled 1 week after the last injection, in order to assess the presence of circulating antibodies. The respiratory sublethal infections were monitored 2 weeks after the second immunization.

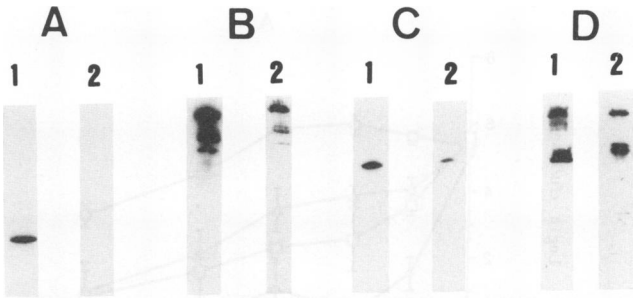

FIG. 1. Characterization of PTX, FHA, pertactin, and AC-Hly in bacterial suspensions of *B. pertussis* and *B. parapertussis*. One-microliter *B. pertussis* (lanes 1) or *B. parapertussis* (lanes 2) bacterial suspensions were subjected to SDS-PAGE (8 to 25% polyacrylamide). After electrophoresis, proteins were transferred to Hybond C-Super membranes. Membranes were incubated with polyclonal sera raised against *B. pertussis* detoxified PTX (A), *B. pertussis* FHA (B), *B. pertussis* pertactin (C), or *B. pertussis* AC-Hly (D). The immunochemical detection was performed with horseradish peroxidase-labelled sheep anti-mouse immunoglobulins by using the Amersham enhanced chemiluminescence system.

Intranasal infection of mice. *B. pertussis* or *B. parapertussis* organisms were grown on BG medium, as described above. The bacteria were resuspended and diluted in 1% Casamino Acids. Sublethal challenges were performed by intranasal injections of 50 μl of bacterial suspensions. Infected mice were sacrificed by cervical dislocation 1 h after exposure (at the time designated day 0) and at various days thereafter (four to six mice per time point). The lungs were removed and homogenized in saline with tissue grinders. Dilutions of lung homogenates were sampled on BG, and CFU were counted after 3 days of incubation at 36°C. All experiments were performed three times with FHA, PTX, AC, and AC-Hly but only one time with pertactin and gave identical results. The data were tested for statistical significance by Student's *t* test.

RESULTS

Immunological detection of *B. parapertussis* FHA, pertactin, and AC-Hly. It is well established that *B. parapertussis* synthesizes factors similar to those of *B. pertussis* (6, 12, 14, 15, 21, 25). As shown in Fig. 1, polyclonal antibodies specific to purified *B. pertussis* FHA, pertactin, and AC-Hly recognized similar factors in *B. parapertussis* bacterial suspensions. This indicates that *B. pertussis* and *B. parapertussis* synthesize factors that cross-react immunologically. However, *B. pertussis* anti-PTX antibodies do not recognize any protein, confirming the nonexpression of this factor by *B. parapertussis* (3).

Protective efficacies of purified *B. pertussis* detoxified PTX, FHA, and pertactin against *B. pertussis* and *B. parapertussis* infections. It was previously shown that purified *B. pertussis* detoxified PTX, FHA, and pertactin are protective antigens against *B. pertussis* infection in the murine respiratory model (11, 29, 31, 34). As shown in Fig. 2A, we confirmed these results; among these factors, FHA and pertactin were significantly more protective against bacterial colonization than PTX, which was also protective but to a lesser extent. We examined, in the present study, their protective efficacies

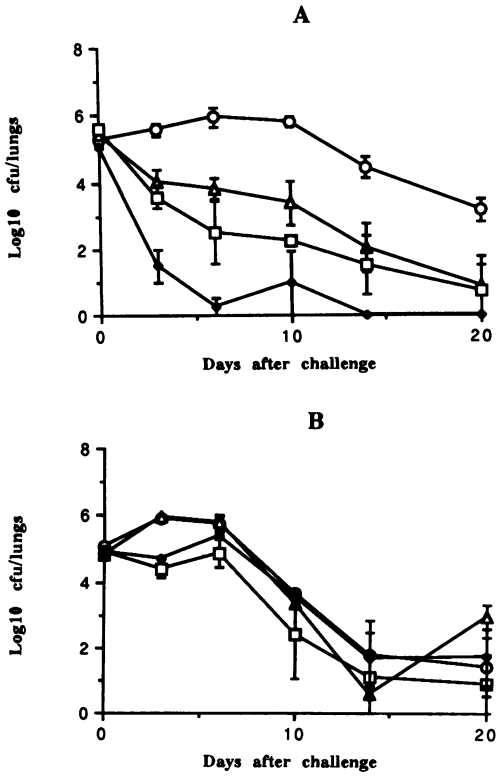

FIG. 2. Protective activities of *B. pertussis* detoxified PTX, FHA, and pertactin against *B. pertussis* and *B. parapertussis* lung colonizations. Mice 3 to 4 weeks old were immunized twice, with a 2-week interval between injections, with 8 μg of *B. pertussis* detoxified PTX (△), FHA (□), or pertactin (◆) adsorbed on aluminum hydroxide, or with buffer containing aluminum hydroxide alone as control (○). They were infected intranasally 2 weeks later with 10^5 CFU of *B. pertussis* Tohama (A) or 10^5 CFU of *B. parapertussis* 63.2 (B). The plots show the geometric standard mean ± standard deviation (bars) for four mice per time point.

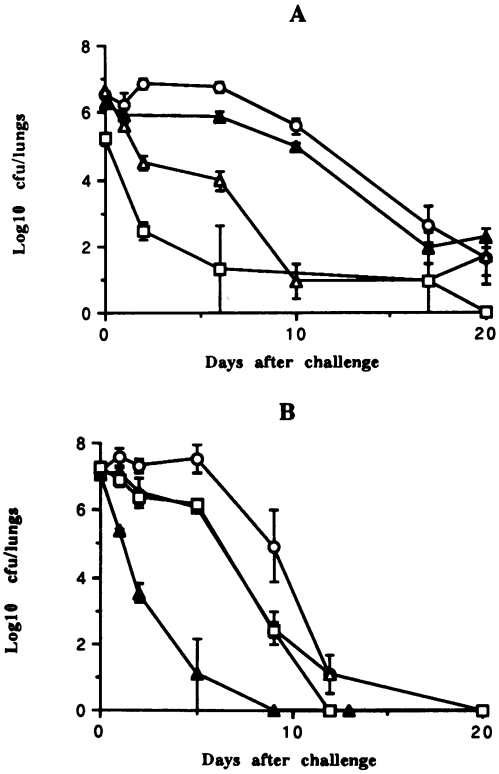

FIG. 3. Protective activities of *B. pertussis* or *B. parapertussis* AC fragments and AC-Hly against *B. pertussis* or *B. parapertussis* lung colonizations. Mice 3 to 4 weeks old were immunized twice, with a 2-week interval between injections, with 4 μg of *B. pertussis* Tohama (△) or *B. parapertussis* 63.2 (▲) AC fragments, 20 μg of *B. pertussis* Tohama AC-Hly (□), or buffer containing aluminum hydroxide alone as control (○). They were infected intranasally 2 weeks later with 5×10^6 CFU of *B. pertussis* Tohama (A) or 10^7 CFU of *B. parapertussis* 63.2 (B). The plots show the geometric standard mean ± standard deviation (bars) for six mice per time point.

against *B. parapertussis* infection. Surprisingly, none of these factors were protective against *B. parapertussis* bacterial colonization. As shown in Fig. 2B, in control mice, immunized only with control buffer, bacterial counts in the lungs increased from 10^5 CFU immediately after challenge to 10^6 CFU on days 3 and 6 after challenge. In mice immunized with pertactin, bacteria were able to adhere to and to multiply in the lungs as did the bacteria in the control mice. In mice immunized with detoxified PTX or FHA, bacterial counts increased in the lungs to 8×10^5 only on day 6 after challenge. The rates of clearance of the bacteria in the lungs were similar in all immunized mice and in control mice. This result was expected for PTX, because *B. parapertussis* does not express this factor; however, it was not expected for the other factors, because this *Bordetella* species synthesizes similar factors that cross-react immunologically (Fig. 1).

Protective efficacies of *B. pertussis* and *B. parapertussis* AC-Hly and AC fragments against *B. pertussis* and *B. parapertussis* infections. We have previously shown that *B. pertussis* AC-Hly and *B. parapertussis* AC-Hly were protective antigens against *B. pertussis* and *B. parapertussis* infections, respectively (16). In the present study, we tested their protective efficacies against both *Bordetella* infections. AC-Hly and AC fragments were purified as described previously (16, 18). The purified AC-Hly preparations we used were devoid of toxic activities because of our purification procedures (16). AC fragments were also devoid of hemolytic and toxic activities because they do not carry the hemolysin part of the molecule (16, 18). We confirmed that *B. pertussis* AC-Hly and AC fragments protected against *B. pertussis* infection. As shown in Fig. 3A, there was no multiplication of bacteria in the lungs of mice immunized with these antigens, compared with control mice, but there was persistence of the bacteria in the lungs of mice immunized with *B. parapertussis* AC fragments. Similar results were obtained when another *B. pertussis* strain was used for the challenge (data not shown). Vice versa, as shown in Fig. 3B, *B. parapertussis* organisms did not multiply but persisted in the lungs of mice immunized with *B. pertussis* AC-Hly and AC fragments, and their rate of clearance was similar to that of the bacteria in the control mice, but *B. parapertussis* organisms were cleared in mice immunized with *B. parapertussis* AC fragments. Thus, despite the high level of homology between AC-Hly and AC fragments from both species, they are immunologically different.

DISCUSSION

B. pertussis and *B. parapertussis* are two species responsible for outbreaks of whooping cough. The diseases induced

by these two species are not easily distinguishable clinically (28). Both species synthesize similar factors, such as FHA, pertactin, dermonecrotic toxin, tracheal cytotoxin, and AC-Hly, with the exception of PTX. However, *B. pertussis* infection and *B. pertussis* vaccination do not seem to protect against *B. parapertussis* infection (20).

In order to improve pertussis vaccination safety and efficacy, efforts to develop a new acellular vaccine are being made. Data from clinical trials showed that new vaccines, containing detoxified PTX or detoxified PTX and FHA, protect against the disease but protect incompletely against infection (1, 36). Therefore, other factors should be included; pertactin and AC-Hly may be good candidates for inclusion in future acellular vaccines. It is well established that these two factors are protective against *B. pertussis* infection in the murine model (16, 34). Since *B. parapertussis* expresses factors that possess a very strong degree of homology with *B. pertussis* factors (more than 93% for pertactin), one could expect that an acellular preparation containing these *B. pertussis* factors would protect against *B. parapertussis* infections. As shown in this study, this may not be the case. We used a murine respiratory model to examine the protective efficacies of four purified *B. pertussis* antigens against *B. parapertussis* infection. None of these factors were able to protect against *B. parapertussis* infection, whereas they protected against *B. pertussis* infection. This result was expected for detoxified PTX but not for the other factors because they possess a very high degree of homology with *B. parapertussis* factors, as shown in previous studies and confirmed in this study. Similar results were obtained with *B. pertussis* AC-Hly and AC fragments. These antigens protected against *B. pertussis* infection but protected weakly against *B. parapertussis* infection. Furthermore, we examined the protective efficacy of one protective *B. parapertussis* antigen against *B. pertussis* and *B. parapertussis* infections. Similarly, *B. parapertussis* AC fragments, which protected against *B. parapertussis* infection, did not protect against *B. pertussis* infection. Moreover, similar results were obtained when mice were infected with other *B. pertussis* strains.

These results suggest that the abilities of protective antigens to stimulate an effective immune response are species specific. The cross-reactivity demonstrated by Western blot analysis most likely reflects reactivity versus conserved domains of the protein, whereas protective domains, most likely to be surface accessible, are antigenically variable between the two species.

In the case of pertactin antigen, both *B. pertussis* (P.69 pertactin) and *B. parapertussis* (P.70 pertactin) sequences are known (11, 21). It was shown that both antigens share more than 93% homology. The major differences occur in the number of repeating units within the two families of repeat motifs. Their functions are not known, but the region spanning the repeating units has been demonstrated to be immunodominant. Our results suggest that these regions may play an important role in protective activity. The other differences are located in the regions flanking the Arg-Gly-Asp motif involved in the adherence of the bacteria to eukaryotic cells. This difference, as emphasized by Li et al. (21), may be important in altering the affinity of binding of P.70 pertactin, compared with P.69 pertactin, or altering the class of integrin receptor to which the different proteins may bind. One can speculate that this difference may also modify the protective activity of the protein and suggests that this region may carry an important protective epitope.

Observations similar to those for pertactin were made with the AC-Hly antigen. It is now well known that this toxin possesses homology with members of the RTX toxin family, including *Escherichia coli* α-hemolysin. Both *B. pertussis* and *B. parapertussis* synthesize a very similar AC-Hly toxin (5, 14, 16), although some differences in molecular weight and enzyme activities were observed (16). To be active, AC-Hly toxin requires posttranslational modification mediated by the protein coded for by the *cyaC* gene (4, 32). We recently obtained data showing that the modified region of AC-Hly is important for AC-Hly protective activity (5a). One can speculate that these regions in *B. pertussis* and in *B. parapertussis* may be slightly different. Furthermore, it has to be noted that *B. parapertussis* contains but does not transcribe the PTX operon (3). The lack of expression is due to a cluster of mutations which affect the efficiency of the promoter. *B. parapertussis* contains structural genes encoding functional proteins, which, if expressed, possess the same ADP-ribosylation activity as the *B. pertussis* PTX (3). However, data from the nucleotide sequence showed that the *B. parapertussis* S1 subunit structural gene contains many base pair substitutions all over the gene compared with the *B. pertussis* gene (2). All of these findings indicate that, despite the high level of homology between both *B. pertussis* and *B. parapertussis* species, no or very little cross-protection is observed, confirming early studies by Lautrop (20) and Linneman and Perry (22). One can predict that pertussis acellular vaccines will not protect against other *Bordetella* infections, *B. parapertussis* and *Bordetella bronchiseptica*, the animal pathogen, which is sometimes a pathogen for humans.

For several years, the goal of ongoing research has been to improve the safety and efficacy of pertussis vaccine. We propose use of a cellular vaccine containing a nontoxic *B. pertussis* strain (i.e., producing nontoxic PTX and nontoxic AC-Hly) and a nontoxic strain of *B. parapertussis*. Introduction of a nontoxic strain of *B. parapertussis* into this vaccine may broaden the scope of activity of the vaccine and also confer protection against *B. parapertussis* infections.

While *B. pertussis* and *B. parapertussis* were thought to share conserved proteins, the present study has shown evidence of the absence of cross-protection and therefore outlines that, as in other gram-negative bacteria, *Bordetella* surface proteins vary immunologically, probably in response to the host immune response.

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