**Bordetella pertussis** Protein Pertactin Induces Type-Specific Antibodies: One Possible Explanation for the Emergence of Antigenic Variants?

Qiushui He,¹ Johanna Mäkinen,¹ Guy Berbers,⁴ Frits R. Mooi,⁵ Matti K. Viljanen,² Heikki Arvilommi,¹ and Jussi Mertsola³

¹Department of Human Microbial Ecology and Inflammation, National Public Health Institute, and Departments of ²Medical Microbiology and ³Pediatrics, University of Turku, Turku, Finland; ⁴Laboratory for Clinical Vaccine Research and ⁵Research Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

Divergence has been found between **Bordetella pertussis** vaccine strains and circulating strains. Polymorphism in pertactin (Prn) is essentially limited to region 1, which is made up of repeats. Today, the 3 most prevalent Prn variants are Prn1–3. Vaccine strains produce Prn1, whereas Prn2 is the predominant type found in circulating strains. We investigated how variation in region 1 affects the production of human serum antibodies. Individuals infected by Prn2 strains had significantly fewer antibodies to Prn1 did than those infected by Prn3 strains and those immunized with a booster dose of acellular vaccines containing Prn1. Moreover, in contrast to vaccine recipients and subjects infected by Prn3 strains, individuals infected by Prn2 strains had hardly any antibodies specific to the variable region of Prn1. These results indicate that conformational changes have occurred in the variable region of Prn, which may offer a possible explanation for the emergence of Prn2 strains in certain countries.

**Bordetella pertussis**, a small gram-negative coccobacillus, is the causative agent of pertussis (whooping cough) in humans. Pertussis is 1 of the 10 most common causes of death from infectious disease worldwide [1]. Immunization is the most effective method for the prevention and control of pertussis and has been used successfully for decades. *B. pertussis* produces various virulence factors, of which filamentous hemagglutinin (FHA), fimbriae, pertactin (Prn), and pertussis toxin (PT) induce protective immunity and are included in the new acellular pertussis vaccines. Acellular pertussis vaccines are replacing the old whole-cell vaccines in the industrialized world.

During the past 10 years, a resurgence of pertussis has been seen in countries with high vaccination coverage of whole-cell vaccines [2–5]. One explanation for this increasing incidence is the antigenic divergence between vaccine strains and circulating strains. Recently, antigenic variation of Prn and PT has been reported in Europe and the United States [6–10]. Prn, a 69-kDa outer-membrane protein, contains 2 immunodominant regions, 1 and 2, composed of repeating units of 5 (GGXXP) or 3 (PQP) amino acids, respectively [11]. Polymorphism in Prn is essentially limited to region 1 [12] and is located adjacent to an RGD (arginin-glycine-aspartic acid) motif implicated in adherence [13].

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Reprints or correspondence: Dr. Qiushui He, Pertussis Reference Laboratory, National Public Health Institute, Kimanlyynkatu 13, 20520 Turku, Finland (qiushui.he@ktl.fi).

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0022-1899/2003/18708-0004$15.00
Today, the 3 most prevalent Prn variants are Prn1–3 [6–10]. The vaccine strains produce Prn1 [12, 14], whereas Prn2 is the predominant type found in circulating strains. In Finland, 72% of the clinical strains isolated between 1990 and 1996 harbored Prn2, 12% harbored Prn3, and only 7% harbored Prn1 [7]. Prn1 and Prn3 are similar, with a difference of only 2 aa, whereas Prn2 has an additional repeat.

The goal of the present study was to investigate how variation in region 1 affected the production of serum antibodies that are believed to be important in protection against pertussis in humans [15–18]. A number of paired serum samples were collected from patients who had been confirmed by culture to be infected by B. pertussis strains expressing Prn2 or Prn3 and from healthy individuals who had received a booster immunization of diphtheria-tetanus–acellular pertussis vaccines (DTPa) containing Prn1. Prn type–specific antibodies and the region 1–specific antibodies were measured by ELISA that used purified Prn1 or synthetic peptides as antigens. The ability of human antibodies to compete with monoclonal antibodies (MAbs) recognizing region 1–dependent epitopes were analyzed by a blocking ELISA.

**MATERIALS AND METHODS**

**Immunization of pertussis in Finland.** Immunization against pertussis was introduced in Finland in 1952 [19]. Since 1958, the vaccine has contained strain 18530. Because serotypes 1 and 2 strains emerged in the 1970s, strain 1772 was introduced to the vaccine (vol/vol) in 1976. Both of the vaccine strains harbor Prn1 [7]. The vaccine is manufactured by the National Public Health Institute and contains formalin-killed B. pertussis organisms per dose, combined with diphtheria and tetanus toxoids. Infants are immunized at ages 3, 4, and 5 years [21]. All of the patients had their cultures confirmed between 1990 and 1998. Of the 41 patients with pertussis, 4 were born before 1952, the year pertussis immunization was introduced in Finland, 5 were born between 1955 and 1970, and 32 were born during the 1980s. Of the 32 patients who were born during the 1980s, 23 patients’ immunization history was known [22, 23]. All had been immunized in infancy with 3 doses of Finnish DTPw vaccine, and 19 had also received a booster dose at age 2 years. All 14 patients with parapertussis had received 4 doses of Finnish DTPw vaccine in infancy. At the time of nasopharyngeal swabbing and blood sampling, 38 patients with pertussis had had a median of 12 days of cough (range, 5–41 days), and the other 3 remained asymptomatic and developed cough later on. The median interval between the 2 blood samples was 27 days (range, 16–84 days). Of the 41 patients with pertussis, 36 were infected by Prn2 strains and 5 by Prn3 strains (confirmed by polymerase chain reaction [PCR]–based sequencing of pertactin gene) [7]. No statistically significant difference was found among age, days having cough at the first blood sampling, and interval between the 2 blood samples between patients infected by B. pertussis strains Prn2 and Prn3 ($P = .281$, $P = .704$, and $P = .426$, respectively). At the time of nasopharyngeal swabbing and blood sampling, 7 patients with parapertussis had had cough, and the other 7 remained symptom free. The interval between the 2 blood samples taken from patients with parapertussis was 28–56 days.

**Bacterial culture and DNA sequencing.** Details of bacterial culture, DNA extraction, and PCR-based sequencing of the Prn gene have been described elsewhere [6, 7].

**Peptide synthesis.** The 3 linear peptides were designed to represent the variable region of Prn1, Prn2, and Prn3, respectively. The amino acid sequences are shown in figure 1A. The peptides—PeP1, PeP2, and PeP3—were synthesized by the MedProbe representing Prn1 (271–290), Prn2 (271–295), and Prn3 (271–290), respectively.

**Production of MAbS.** Details of the production of MAbs have been described elsewhere [24]. The purified Prn1, kindly provided by GSK and Chiron-Biocine, were used to raise the MAbs [24]. Three MAbs, PeM1, PeM4, and PeM5, were used in the present study. PeM4 has been proved to bind to epitope GGFPGGFGFP, representing 2 repeats on region 1, whereas PeM1 and PeM5 [24] were known to recognize conformational epitopes of the variable region. All 3 MAbs have been shown...
to provide protection against intranasal challenge in BALB/c mice with *B. pertussis* [24].

**Determination of serum IgG antibody.** The determination of serum IgG antibodies was performed by ELISA, as described elsewhere [22]. The coating antigens were the purified proteins Prn1, PT, or FHA, kindly provided by GSK, or the synthetic linear peptides (figure 1A). To make sure that the ELISA using peptides as coating antigens worked, the reaction conditions, including the concentration of peptides used and the incubation time and temperature of microtiter plate, were first optimized. The concentration of these antigens used was 5 μg/mL. Test serum samples were diluted to 1:100 or 1:50 where appropriate. Results were expressed as relative ELISA units (1 ELISA unit, 1:100 of the corresponding antibody concentration in the standard serum). The standard serum that made up highly positive pool serum samples, moderately positive pool serum samples, and negative pool serum samples was included in each assay. All samples were tested in duplicate. For the ELISA using peptides as antigens, only paired serum samples obtained from 40 adolescent vaccine recipients and 15 subjects randomly selected from 36 Prn2-infected patients were tested.

**Blocking ELISA.** The blocking of MAb binding to Prn1 by human serum antibodies was determined by ELISA. The concentration of Prn1 was 5 μg/mL. After the wells were blocked with PBS supplemented with 1% normal sheep serum (NSS-PBS), the test serum samples, diluted in 1:100 in NSS-PBS, were added. After a 2-h incubation at 37°C, MAbps were added. The relative binding of MAb to Prn1 was calculated as absorbance of test serum/absorbance of NSS-PBS.

**Statistical analysis.** The Mann-Whitney *U* test was used to compare data between the 2 groups. *P* < .05 was considered to be statistically significant.

**RESULTS**

We first wanted to investigate whether there is a type-specific immune response against Prn. Therefore, the reactivity of serum samples from individuals infected or vaccinated with different Prn variants was tested against Prn1 (figure 1B), the only currently available purified Prn. The antibody response to other important antigens of *B. pertussis* such as PT and FHA in humans was also investigated (figure 1C). We recruited patients from whom *B. pertussis* strains harboring Prn2 or Prn3 had been isolated. Two serum samples from each patient were collected, with a median interval of 27 days, and were tested by ELISA that used Prn1 as the coating antigen. Because the circulating strains harboring Prn1 were rare (7%) in Finland during the 1990s [7], patients from whom *B. pertussis* Prn1 strains had been isolated and their serum samples were not available. Instead, we recruited children who had been immunized in infancy and had received boosters at age 2 years with DTPa

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**Figure 1.** Sequence of the pertactin (Prn) region 1 and human antibody responses. A. Amino acid sequences of region 1 of different pertactin types of *Bordetella pertussis* and *Bordetella parapertussis*. Prn1, Prn2, and Prn3 differ only in region 1. Prn1 and Prn3 are similar with a difference of only 2 aa (278 and 279), whereas Prn2 has an additional repeat of 5 aa (291–295). *B. parapertussis* Prn also differs from *B. pertussis* Prn outside region 1. The underlined sequences were used as synthetic peptides in ELISA.

**B.** Serum IgG antibody responses to Prn1 in different study subjects. For the vaccine recipients, serum samples were taken before and 1 month after immunization; for the patients, the median interval between the 2 serum samples was 27 days. *P* < .05; **P** < .01; ns, Not significant. **C.** Serum IgG antibodies to pertussis toxin (PT) and filamentous hemagglutinin (FHA). **D.** Serum IgG antibodies to the synthetic peptides. Pep1, Pep2, and Pep3 were derived from region 1 of Prn1, Prn2, and Prn3, respectively. Only paired serum samples obtained from 40 adolescents who received a booster dose ofacellular vaccine containing Prn1 and 15 subjects randomly selected from 36 Prn2-infected patients were tested. *P* < .05, except where marked; otherwise, no statistically significant difference was found in antibody responses among the paired serum samples of the responders, the nonresponders, and the patients.
that contained PT, FHA, and Prn1 and adolescents who had been immunized in infancy with Finnish whole-cell vaccine that contained 2 Prn1 strains and had received boosters of an acellular vaccine that contained PT, FHA, and Prn1 [20, 21]. Their serum samples were collected before and 1 month after the booster immunization. In addition, serum samples taken from patients who were culture positive for B. parapertussis were also tested. No variation has been found in the region 1 of these B. parapertussis strains isolated (figure 1A).

A significant increase was found in the level of IgG antibody to Prn1 in patients from whom B. pertussis strains harboring Prn2 or Prn3 were isolated and in children and adolescents after a booster dose of immunization (figure 1B). The level of antibody to Prn1 was similar in the first serum samples of the patients with strains harboring Prn2 or Prn3 (P = .353; figure 1B). However, significantly lower levels of anti-Prn1 antibodies were found in the second serum samples of the patients with strains harboring Prn2 than those with strains harboring Prn3 (P = .037) and than children (P < .001) or adolescents (P < .001) after the booster immunization (figure 1B). No significant difference was found in the level of anti-PT and -FHA antibodies in both first (P = .857 and P = .357) and second serum samples (P = .150 and P = .889) of the patients with strains harboring Prn2 or Prn3, respectively (figure 1C). Children primed and boosted with tricomponent DTPs had the highest antibody responses against PT, FHA (data not shown), and Prn1.

We then tested whether there are region 1–specific antibodies in human serum. Three linear synthetic peptides, Pep1, Pep2, and Pep3, representing region 1 of Prn1, Prn2, and Prn3, were used as coating antigens in ELISA (figure 1A). Of the 40 adolescents who received a booster dose of acellular vaccine containing Prn1, 6 (15%) had an increase in or high level of antibody to the peptides (figure 1D). However, this increase or high level found in the 6 subjects was observed for all 3 of the peptides. Of the 15 subjects randomly selected from 36 patients whose strains harbored Prn2, only 1 had a marginal increase in antibody response to all 3 peptides (figure 1D). These results indicate that the region 1–specific antibodies are rarely developed after infection or immunization when tested with the linear peptides as antigens and that these antibodies may not be able to distinguish the variable regions. On the other hand, the antibodies specific to the variable region may mainly recognize the conformational epitopes than those linear ones used in the present study [24].

Next, a blocking ELISA was developed to test the ability of human antibodies to compete with MABs, which are known to bind to region 1–dependent epitopes [24]. Three MABs, PeM1, PeM4, and PeM5, were used: PeM4 has been proved to bind to a linear epitope in region 1, whereas PeM1 and PeM5 were shown to recognize conformational region 1–dependent epitopes. Purified Prn1 was used as the coating antigen.

Human IgG antibodies developed after infection or a booster immunization were used in the blocking assay, and a blocking activity that was able to block 50% of MABs binding to Prn1 was defined as significant. Only 9%–6% of the patients infected with Prn2 strains developed antibodies that significantly inhibited PeM5 or PeM1 binding to Prn1 (table 1; figure 2), whereas 40%–60% of patients infected by Prn3 strains or 30%–84% of subjects after a booster immunization had developed antibodies that significantly inhibited PeM5 or PeM1 binding (table 1; figure 2). Children primed and boosted with DTPa had the highest responses. Binding of PeM4 to Prn1 was not inhibited by human antibodies after either immunization or pertussis infection (table 1; figure 2). Binding of PeM1, PeM4, and PeM5 to Prn1 was not inhibited by human antibodies after B. parapertussis infection (figure 2).

### DISCUSSION

Prn1 elicits protective immunity in both animals and humans [15–18], and region 1 has been found to be 1 of the 2 immunodominant regions of this protein [11]. Prn1 is included in most of the new acellular pertussis vaccines, and the level of IgG antibody to Prn1 has been found to be associated with a lower likelihood of acquiring pertussis in vaccine efficacy trials [15, 16]. To our knowledge, ours is the first study to show biological effects in humans in relation to the earlier found variation of Prn. The changes in the variable region were found to affect the production of human IgG antibodies to Prn1 in the patients infected by Prn2 strains but not the antibodies to other B. pertussis antigens, such as PT or FHA. The effect observed so far was restricted only to

<table>
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<th>Study</th>
<th>Subjects with antibodies capable of inhibiting &gt;50% of the binding of monoclonal antibodies (MABs) to pertactin (Prn1).</th>
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<tr>
<td>Patients with pertussis</td>
<td>Subjects with antibodies capable of inhibiting &gt;50% MABs binding, %</td>
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<tr>
<td>Prn2b (n = 36)</td>
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<td>Prn3c (n = 5)</td>
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<td>Child (n = 37)</td>
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<td>Adolescent (n = 40)</td>
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- Prn2b and Prn1 recognize a region 1–dependent conformational epitope, whereas Prn4 recognizes a region 1–dependent linear epitope.
- All were culture positive for Bordetella pertussis expressing Prn2.
- All were culture positive for B. pertussis expressing Prn3.
- P < .01 vs. patients infected with B. pertussis Prn2 strains.
- Prn2b, Prn1, and Prn3 expressed in humans in relation to the earlier found variation of Prn. The changes in the variable region were found to affect the production of human IgG antibodies to Prn1 in the patients infected by Prn2 strains but not the antibodies to other B. pertussis antigens, such as PT or FHA. The effect observed so far was restricted only to

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the strains harboring Prn2, currently the predominant strains circulating in both Europe and the United States. In Finland, 72% of the clinical strains isolated between 1990 and 1996 harbored Prn2, and 12% harbored Prn3 [7]. In the United Kingdom, of 105 strains isolated from 1998 to 1999, 47% expressed Prn1, 50% expressed Prn2, and 3% expressed Prn3 [10]. In the United States, of 92 strains isolated between 1989 and 1999, 30% harbored Prn1, and 70% harbored Prn2 [9]. In the present study, we have not measured anti-Prn2 antibodies because of the unavailability of Prn2 antigens.

Region 1 consists of GGXXP repeats and is located adjacent to an RGD motif that has been implicated in adherence [13]. The crystal structure of Prn1 indicates that region 1 forms a loop protruding from a β-sheet [25]. It is conceivable that the shape of this loop is affected by the number of repeats. Of the 3 prevalent Prn types, Prn2 has 1 additional repeat, compared with Prn1 and Prn3, whereas the difference between Prn1 and Prn3 is composed of only 2 aa. The observed effect on the antibody production of humans after infection with strains harboring Prn2 but not Prn3 is in line with its 3-dimensional structure. The type specificity of the immune response is further supported by the evidence that the binding of MAbs that recognize region 1–dependent epitopes to Prn1 was inhibited by human antibodies after immunization with acellular vaccine containing Prn1 or after natural infections caused by B. pertussis strains harboring Prn3 but not Prn2. The observation that the binding of MAbs recognizing region 1–dependent epitopes to B. pertussis Prn1 was not inhibited by human antibodies after natural infections caused by B. parapertussis strains (figure 2) is also consistent with the structural data known for B. pertussis Prn1. The region 1 sequence of B. parapertussis Prn is 1 repeat shorter than Prn1. No variation has been found in this region of B. parapertussis [26].

Our results, which are based on the serum samples from patients with pertussis and from vaccinated subjects, further confirm that MAbs that recognize the conformational region 1–dependent epitopes can compete with human antibodies [24], whereas those bound to a linear epitope do so less or not at all, which suggests that infection caused by Prn2 strains does not induce antibodies against region 1–related epitopes of Prn1. However, these results do not exclude the possibility that there might be other protective epitopes on Prn1 and/or Prn2.

It has also been shown, in the respiratory mouse model, that the tricomponent DTPa vaccine (Infanrix) was highly effective against strains expressing Prn1, Prn2, or Prn3 [14]. However, the Dutch whole-cell pertussis vaccine protected mice against B. pertussis isolates with Prn1 significantly better than those carrying non–vaccine-type Prns [24]. During the past 10 years, a resurgence of pertussis has been seen in countries with high whole-cell pertussis vaccination coverage [2–5]. The possibility that some whole-cell pertussis vaccines used are not very effective cannot be excluded. Acellular pertussis vaccines are currently replacing traditional whole-cell vaccines in the industrialized world. Therefore, it is extremely important to elucidate whether acellular vaccines provide equal protection against B. pertussis representing vaccine or nonvaccine type strains, although it is comforting to know that all efficacy trials have been performed in places with Prn1, Prn2, and Prn3 circulating strains.

It is known that pertussis is a multifactorial disease, and several known virulence factors are involved in its pathogenicity. The protective antibodies induced by immunizations or natural infections wane with time. It has been shown that antibodies against Prn1 persist longer than those against other B. pertussis antigens, such as PT. The antibodies to Prn1 play a role in protection against pertussis but may not be decisive when the level of antibodies to other antigens (e.g., PT) is high. When other antibodies decrease to low or undetectable levels, the role played by antibodies to Prn1 in protection would become more decisive, because these antibodies persist longer [20, 27]. The role of immunologic memory is likely to be important as well. The type specificity of the immune response induced by Prn suggests that infecting strains that harbor Prn2 do not
induce antibodies against the conformational epitopes that are induced by vaccine strains expressing Prn1 and Prn3 and might explain the emergence of \emph{B. pertussis} strains with Prn2.

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\textbf{References}