Synergic Effect of Genotype Changes in Pertussis Toxin and Pertactin on Adaptation to an Acellular Pertussis Vaccine in the Murine Intranasal Challenge Model*

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The Bordetella pertussis pertussis toxin and pertactin (Prn) are protective antigens and are contained in acellular pertussis vaccines. Polymorphisms in the A subunit of pertussis toxin (PtxA) and pertactin have been proposed to mediate vaccine resistance and contribute to pertussis reemergence. To test this hypothesis, previous studies compared clinical isolates expressing different alleles for the proteins. However, other virulence factors or virulence factor expression levels also may vary, confounding the analysis. To overcome these limitations, we constructed isogenic mutants of B. pertussis Tohama expressing the alleles ptxA1 or ptxA2 and prn1 or prn2 and compared the efficacies of an acellular pertussis vaccine against the mutants in a mouse model. While the vaccine was effective against all of the B. pertussis strains regardless of the allele expression pattern, the strain expressing ptxA1 and prn2 displayed a survival advantage over the other strains. These results suggest that an allele shift to the ptxA1 prn2 genotype may play a role in the emergence of pertussis in vaccinated populations.

Pertussis (whooping cough) is a highly contagious disease caused mainly by Bordetella pertussis. Since the introduction of efficacious pertussis vaccines in the 1940s and vaccination programs on a global scale, the incidence of and deaths due to pertussis have decreased dramatically around the world (36). However, within the last few decades, pertussis resurgence has occurred, even in countries with high vaccine coverage (8, 31). Reemergence has been proposed to be due to several factors, e.g., waning immunity from childhood vaccination in adolescents and adults, improved methods for pertussis diagnosis, and increased awareness of pertussis among medical professionals and the public. However, it has also been proposed that reemergence may be due to antigenic shift in the circulating strains in response to pertussis vaccines. Previous studies (11, 23, 31, 35) reported that polymorphisms of the pertussis toxin A subunit (PtxA) and pertactin (Prn) in circulating B. pertussis strains in the Netherlands distinguished these proteins from those present in the pertussis vaccine (8, 30), and these polymorphisms have now been detected worldwide (1, 3, 4, 6, 8, 12, 19, 26, 30). Since these two proteins have been shown to be protective antigens of B. pertussis and are used as essential components of acellular pertussis vaccines, it has been proposed that the polymorphisms may confer vaccine resistance and contribute to reemergence. Indeed, there are differences in alleles of ptxA and prn between seed strains for manufacturing pertussis vaccines and currently circulating strains in most countries (8, 30). Specifically, most vaccine seed strains have ptxA2 and prn1 alleles (Fig. 1), but in many countries the dominant alleles in recently circulating strains are ptxA1 and prn2 (8). Since circulating strains expressing ptxA1 and prn2 are dominant or are increasing in prevalence in most countries, it is likely that these strains are the most successful clones in mass-vaccinated populations (19). The National Institute of Infectious Diseases in Tokyo, Japan, has been reporting increases in pertussis cases in Japan since 2007 (25). National surveillance data show that increases in pertussis cases have occurred among all age groups except children less than 1 year old (25). The most remarkable increase has occurred among adults (individuals ≥20 years old); however, a significant increase even in the vaccinated 1- to 9-year-old population has been recorded (25). A few studies have described antigenic shift in circulating strains in Japan. Guiso et al. reported that the rate of recovery of non-vaccine-type clinical isolates shifted from 0% for the years 1979 to 1991 to 28.6% for the years 1992 to 1996 (9). Recently, Han et al. determined that non-vaccine types account for 43.3% of the clinical isolates collected between the years 1992 and 2007 (13). These observations may suggest that antigenic shift in circulating strains from vaccine type to non-vaccine type has contributed to the recent pertussis resurgence.

Several studies have attempted to clarify the relationship between the antigenic shift and vaccine efficacy by using epidemiologic and experimental approaches (1–3, 6, 8, 12, 14, 16, 19, 20, 23, 26, 30, 31); however, these studies have been controversial due to the correlative nature of the analyses. Previous studies used clinical isolates to determine if polymorphisms could confer resistance to vaccine efficacy (1, 2, 16). However, B. pertussis produces several kinds of virulence factors, including toxins and adhesins. Polymorphisms in genes other than ptxA and prn have been reported (29, 32), and these differences may be responsible for increased fitness of the organism. To avoid this problem, we constructed isogenic mu-
tants of the *B. pertussis* Tohama strain that expressed different combinations of the *ptxA* and *prn* alleles and we examined the protective efficacy of a commercial acellular pertussis vaccine against each of the mutants in a murine intranasal challenge model. Our results suggest that alleles of *ptxA* and *prn* can influence the protective efficacy of pertussis vaccines and that continuous surveillance of the genotypes of *B. pertussis* should be performed.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** A streptomycin-resistant derivative of *B. pertussis* strain Tohama (15) and strain CCHMC1, which was isolated at Cincinnati Children’s Hospital Medical Center (Cincinnati, OH) in 2005, were used in this study. DNA sequencing of the alleles of *ptxA* and *prn* of both strains was performed as described by Mooi et al. (22). Tohama expresses *ptxA2* and *prn1*, and CCHMC1 expresses *ptxA1* and *prn2*. We used CCHMC1 as the donor of *ptxA1* and *prn2* alleles. For routine propagation, *B. pertussis* was grown on Bordet-Gengou agar (BD Biologics, Sparks, MD) supplemented with 1% glycerol and 15% defibrinated sheep blood (BG agar) at 36°C for 4 days. Antibiotics (i.e., streptomycin at a concentration of 100 μg/ml and ampicillin at a concentration of 200 μg/ml) were added to BG agar plates when appropriate. *Escherichia coli* DH5α and SM10pir were used for cloning and construction of *B. pertussis* mutants. *B. pertussis* was grown on Bordet-Gengou agar (BD Biologics, Sparks, MD) supplemented with 1% glycerol and 15% defibrinated sheep blood (BG agar) at 36°C for 4 days. Anti-biotics (i.e., streptomycin at a concentration of 100 μg/ml and ampicillin at a concentration of 200 μg/ml) were added to BG agar plates when appropriate. *Escherichia coli* DH5α and SM10pir were used for cloning and construction of *B. pertussis* mutants (18). *pDONR221* (Invitrogen, Carlsbad, CA) and pABB- CRS2 were used as vectors.

**Generation of *B. pertussis* Tohama mutants.** Homologous recombination was used to construct mutants as described previously (18), with modified mating agar (28). In brief, the region containing the full-length *ptxA1* gene of the CCHMC1 strain was amplified by using primers PTXA-F (GCCCGCGGTGCT GGCGCGTGTTTCGCGCACGACTCT) and PTXA-R (AATGACCGGTTGCCCGCATA) with H11032 and amplification of the region containing the whole *prn2* gene of the CCHMC1 strain was performed using PRN-F (GGCACAGGACC GGGCGGTGTTTCGCGCACGACTCT) and PRN-R (GGCGTGGTGCGCCT TAAAGGGCGGTGACCCACTTA) with attB adaptors. The PCR products were cloned into *pDONR221* to obtain *pDONR-PTXA1* and *pDONR-PRN2* by site-specific recombination techniques using the Gateway cloning system (Invitrogen). The regions transferred into the *pDONR221* plasmid were sequenced for verification. *pDONR-PTXA1* or *pDONR-PRN2* was mixed with pABB-CRS2 to obtain pABB-PTXA1 and pABB-PRN2 by using the Gateway cloning system. pABB-PTXA1 or pABB-PRN2 was introduced into *E. coli* SM10pir and mobilized into the *B. pertussis* strain Tohama by conjugation. The resulting mutant strains were designated Tohama (*ptxA1 prn1*) or Tohama (*ptxA2 prn2*). pABB-PTXA1 was transconjugated into Tohama (*ptxA2 prn2*) to generate Tohama (*ptxA1 prn2*). Sequences of the regions around the *ptxA* and *prn* alleles were determined to verify appropriate homologous recombination.

**B. pertussis growth curves and production of PT and Prn.** *B. pertussis* grown on BG agar for 4 days was streaked onto BG agar plates. After 24 h, the cells were used to inoculate Stainer-Scholte broth to an optical density at 650 nm (OD<sub>650</sub>) of 0.1 and the broth was incubated as a static culture at 36°C for 96 h. The OD<sub>650</sub> was measured every 12 h. The cultures were centrifuged, and pertussis toxin (PT) in supernatants was measured by the Chinese hamster ovary cell-clustering assay described by Gillenius et al. (7). PT activity in the supernatants of cultures was expressed as the maximum dilution that induced cell clustering. Prn in cell pellets was detected by Western blotting of samples of 2.5 × 10<sup>5</sup> cells/lane, performed as described previously (28). Anti-Prn rabbit serum, prepared at the Kitasato Institute, Tokyo, Japan, and alkaline phosphatase-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories) were used to detect Prn. Prn production was quantified with densitometry software (Image Quant TL; GE Healthcare, Buckinghamshire, United Kingdom).

**Vaccine.** Commercial pediatric diphtheria-tetanus-acellular pertussis (DTaP) vaccine lot AC009A manufactured by the Kitasato Institute was used. The vaccine was prepared from a culture of the wild-type Tohama strain (*ptxA2 prn1*) and contained formalin-detoxified PT (1 μg protein nitrogen/single human dose [1 μg PN/SHD]), filamentous hemagglutinin (4.5 μg PN/SHD), and Prn and fimbriae (0.5 μg PN/ SHD). The vaccine had a potency of approximately 14 U/SHD in the modified intracerebral challenge assay used as a national certification test for pertussis vac-

**Immunization and intranasal infection.** An intranasal challenge model was used to evaluate the protective efficacy of the DTaP vaccine against *B. pertussis* Tohama and its mutants (10). In brief, 3-5-week-old female BALB/c mice (Japan SLC, Hamamatsu) were immunized by two subcutaneous injections of 0.25 SHD (0.125 ml) over a 2-week interval. Two weeks after the second immunization, 50 μl of a suspension containing approximately 6 × 10<sup>8</sup> CFU of *B. pertussis* was instilled intranasally into mice anesthetized by intraperitoneal injection with pentobarbital sodium (Nembutal; Abbott Laboratories, Abbott Park, IL). Two hours (day 0) or 2, 5, or 8 days after the challenge, the mice were euthanized by pentobarbital injection. The lungs of the mice were dissected and homogenized in 10 ml of phosphate-buffered saline (PBS) per lung in a Tetlon homogenizer on ice. After appropriate dilution of samples (10<sup>−1</sup>, 10<sup>−3</sup>, and 10<sup>−5</sup>) with ice-cold PBS, 100-μl aliquots of the three dilutions were spread onto BG agar plates (two plates were used for each dilution). The plates were incubated at 36°C for 4 days. At each time point, colonies of *B. pertussis* on each plate were counted. Usually, we used counts from plates that appeared to have 30 to 300 colonies for calculation of the number of CFU per lung. Once the mean number of CFU per lung was determined, the data for each mouse were log transformed and the arithmetic mean of the transformed data and the standard deviation (SD) for each group were calculated. This study was approved by the Animal Research Committee of Kitasato University and conducted according to the guidelines of the Ministry of Education, Culture, Sports, Science, and Technology.

**Statistical analysis.** The statistical significance of differences between results for different groups was examined by appropriate statistical tests with GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA).

**RESULTS**

**Characterization of the *B. pertussis* mutants.** Samples from *B. pertussis* Tohama mutants expressing different combinations of *ptxA* and *prn* alleles (Fig. 1) were sequenced to confirm the presence of the appropriate alleles of *ptxA* and *prn* (data not shown). The allele switching did not affect the SDS-PAGE profiles for whole-cell lysates (Fig. 2), bacterial growth in

**FIG. 1.** Sequence comparison of the *ptxA* and *prn* alleles.

**FIG. 2.** Comparison of SDS-PAGE profiles between *B. pertussis* strain Tohama (wild type; *ptxA2 prn1*) and the mutant expressing *ptxA1* and *prn2*. Cell lysates of each strain were used for SDS-PAGE as described in Materials and Methods.
Stainer-Scholte broth (Fig. 3), PT production (Fig. 4A), or Prn production (Fig. 4B) for the mutant expressing the ptxA1 and prn2 alleles and the other mutants.

**Efficacy of the vaccine against the wild-type Tohama strain and the mutants in a murine intranasal challenge model.** Using the murine intranasal challenge model described by Guiso et al. (10), we evaluated the efficacy of the DTaP vaccine against the wild-type Tohama strain and the mutants. Mice immunized with the DTaP vaccine were challenged with each *B. pertussis* strain, and the clearance of *B. pertussis* from the lungs of immunized mice was compared with that from the lungs of unvaccinated control mice. *B. pertussis* was recovered at high levels (>10⁶ CFU per lung) from the unvaccinated control mice at all time points after challenge (Fig. 5A). In contrast, *B. pertussis* levels in the vaccinated mice decreased to approximately 1 × 10⁷ to 3 × 10⁴ CFU by 2 days after challenge (Fig. 5B), and significantly fewer bacteria (*P < 0.05 by one-way analysis of variance [ANOVA] and Dunnett’s test) were recovered from the vaccinated mice than from the unvaccinated controls at all time points tested (2, 5, and 8 days postchallenge). These results suggest that the DTaP vaccine was effective against all strains used in this study, regardless of the ptxA allele and the prn allele.

The DTaP vaccine confers less protection against the mutant *B. pertussis Tohama (ptxA1 prn2)* than against the wild type. Relative levels of *B. pertussis* strains recovered from the lungs of immunized mice were assessed. Levels of wild-type Tohama (*ptxA2 prn1*) recovered on days 0, 2, 5, and 8 were 10⁷.0, 10⁴.9, 10³.9, and 10¹.5 CFU/lung, respectively. Recovery of the mutants differing by a single allele, Tohama (*ptxA1 prn1*) and Tohama (*ptxA2 prn2*), was not significantly different from that of wild-type Tohama (Fig. 5B) (*P > 0.05 by two-way ANOVA and Bonferroni’s test). However, levels of Tohama (*ptxA1 prn2*) recovered on days 5 and 8 after infection (10⁵.9 and 10⁴.9 CFU, respectively) were significantly higher than those of wild-type Tohama (10⁵.9 and 10⁴.9 CFU, respectively; *P < 0.05 and <0.001, respectively, by Bonferroni’s test) (Fig. 5B). These results suggest that single mutation of the ptxA allele (from *ptxA2* to *ptxA1*) or the prn allele (from *prn1* to *prn2*) is insufficient for adaptation to the vaccine in the mouse model. In contrast, a double mutation, i.e., from *ptxA2* to *ptxA1* and from *prn1* to *prn2*, made *B. pertussis* better adapted to the vaccine.

We determined the dose-response relationship for the vaccine in the mouse model with wild-type *B. pertussis Tohama (ptxA2 prn1*) and the *ptxA1 prn2* mutant. In brief, mice were immunized twice with 0.0025, 0.025, and 0.25 SHDs of the DTaP vaccine over an interval of 2 weeks. Two weeks after the second immunization, the mice were challenged with wild-type Tohama, and 8 days later, the numbers of CFU in the lungs of the mice were determined. Dose-dependent efficacy was observed and protection, as demonstrated by significant reduction in bacterial recovery, was obtained for vaccine doses between 0.025 and 0.25 SHDs/mouse (Fig. 6). Two-way ANOVA analyses showed that differences in strains and doses of the vaccine affected the numbers of CFU in the lungs (*P < 0.0008 and 0.0001, respectively*). Nonlinear regression analysis was performed. *r²* values for curves for wild-type Tohama and the mutant were 0.98 and 0.96, respectively. The half-maximal inhibitory doses (ID₅₀, based on the dose versus the log transformed number of CFU per lung) of the vaccine for the wild-
type Tohama strain and the mutant were calculated as 0.029 and 0.179 SHDs, respectively. These observations show that Tohama (ptxA1 prn2), expressing two non-vaccine-type alleles, was better adapted to the vaccine than wild-type Tohama, expressing the vaccine-type alleles.

**DISCUSSION**

It has been reported that circulating *B. pertussis* strains have evolved new genetic polymorphisms. As vaccine coverage increases, vaccine-mediated immunity may induce selection for strains that are less affected by the vaccine. The genetic variability of circulating *B. pertussis* isolates appears to be greater during periods of low vaccine coverage than during periods of high vaccine coverage (19, 33). These observations suggest that vaccination may induce clonal expansion of strains which have a selective advantage in vaccinated human populations. Mooi and colleagues reported polymorphisms in two vaccine antigen genes, ptxA and pmr (23, 31). Recently, the frequencies of ptxA1 and pmr2, the non-vaccine-type alleles, have been increasing, and these alleles have become dominant in many countries (1, 3, 4, 6, 8, 12, 19, 26, 30). It is reasonable to hypothesize that *B. pertussis* strains with ptxA1 and pmr2 may have a survival advantage in populations vaccinated with strains expressing the ptxA2 and pmr1 alleles. This hypothesis has been examined using epidemiological data or experimental mouse models and clinical isolates expressing various alleles of ptxA and pmr (1–3, 6, 12, 14, 16, 19, 20, 23, 26, 30, 31). However, *B. pertussis* produces many virulence factors, and clinical isolates may differ in other properties, in addition to having differences in ptxA and pmr. To avoid this problem, we constructed isogenic mutants of *B. pertussis* strain Tohama. The Tohama strain has been used as a seed strain for pertussis vaccines in Japan and Germany (8) and as a reference strain in pertussis research. Also, its whole genome has been sequenced (27). Wild-type Tohama expresses ptxA2 and pmr1. We constructed three Tohama mutants, Tohama (ptxA1 pmr1), Tohama (ptxA2 pmr2), and Tohama (ptxA1 pmr2).

The murine intranasal challenge model has been shown previously to be correlated with vaccine efficacy in humans (10, 21). Using this model, we confirmed that the DTaP vaccine was effective against all of the allelic variants of Tohama. These results suggest that the commercial pertussis vaccine is effective against strains expressing either ptxA1 or ptxA2 and either pmr1 or pmr2. While strains expressing a single non-vaccine-type allele were controlled as effectively as wild-type Tohama, Tohama (ptxA1 pmr2), which expressed two non-vaccine-type alleles, was able to achieve significantly higher levels of colonization in vaccinated mice than strains with one non-vaccine-type allele, suggesting a synergic effect of the changes in both ptxA and pmr alleles. The results suggest that antigen mismatch may be one of the factors causing resurgence of pertussis in vaccinated populations.

The ID$_{50}$ of the pertussis vaccine for the wild-type strain,
expressing efficacy. Improvement of the acellular pertussis vaccines may change in lescent and adult populations whose immunity to pertussis has mismatch shortens the duration of immunity, strains with an- tigens that do not match those in the vaccine may play an important role in increasing pertussis cases, especially in ado- lescent and adult populations whose immunity to pertussis has waned.

Our data suggest that continuous surveillance for antigenic change in B. pertussis is required to maintain pertussis vaccine efficacy. Improvement of the acellular pertussis vaccines may be worthwhile. Inclusion of detoxified PT and Prn from strains expressing ptxA1 and ptm2 could be considered. In addition, adenylate cyclase toxin has been reported previously to help increase the efficacy of an acellular pertussis vaccine (5) and should be considered for inclusion as a vaccine antigen.

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