Live Attenuated Pertussis Vaccine BPZE1 Protects Baboons Against *Bordetella pertussis* Disease and Infection

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Evidence suggests that the resurgence of pertussis in many industrialized countries may result from the failure of current vaccines to prevent nasopharyngeal colonization by *Bordetella pertussis*, the principal causative agent of whooping cough. Here, we used a baboon model to test the protective potential of the novel, live attenuated pertussis vaccine candidate BPZE1. A single intranasal/intratracheal inoculation of juvenile baboons with BPZE1 resulted in transient nasopharyngeal colonization and induction of immunoglobulin G and immunoglobulin A to all antigens tested, while causing no adverse symptoms or leukocytosis. When BPZE1-vaccinated baboons were challenged with a high dose of a highly virulent *B. pertussis* isolate, they were fully protected against disease, whereas naive baboons developed illness (with 1 death) and leukocytosis. Total postchallenge nasopharyngeal virulent bacterial burden of vaccinated animals was substantially reduced (0.002%) compared to naive controls, providing promising evidence in nonhuman primates that BPZE1 protects against both pertussis disease and *B. pertussis* infection.

**Keywords.** pertussis; live attenuated vaccine; baboon model.

Pertussis, also known as whooping cough, is a severe respiratory illness that can be life-threatening, especially in young infants. Its principal etiological agent is *Bordetella pertussis*, although whooping cough–like syndromes can also be caused by infection with other *Bordetella* species [1]. Pertussis vaccination is currently the most effective control measure against whooping cough [2], but despite high global vaccination coverage of >85% [3], pertussis has not been eliminated. Instead, it has recently made a dramatic comeback in several countries, including the United States [4].

The reason for this resurgence may be multifactorial, including increased awareness, improved diagnostics, a mismatch of circulating and vaccine strains, waning vaccine-induced immunity, especially after vaccination with acellular vaccines, and failure of vaccines to protect against asymptomatic colonization and transmission [5]. A recent mathematical modeling study concluded that the most parsimonious explanation for the resurgence of pertussis is asymptomatic transmission of the causative agent [6], and evidence from studies around the world indicates that human subclinical nasopharyngeal *B. pertussis* infections are common [7–10]. Therefore, the reservoir of *B. pertussis* is much larger than previously appreciated, which not only serves as a source of transmission, but may also lead to the development of escape mutants under vaccine selection pressure.

Unlike vaccines against other infectious diseases such as measles, pertussis vaccines have not substantially changed the periodicity of disease incidence [11], suggesting that neither first-generation whole-cell nor second-generation acellular vaccines have effectively controlled *B. pertussis* circulation. The effect of vaccination on asymptomatic colonization and transmission has long been difficult to assess in animal models, as most pertussis vaccine studies have been done on mouse lung colonization models [12]. Mice cannot transmit *B. pertussis*, and both whole-cell and acellular vaccines protect mouse lungs from *B. pertussis* colonization, thereby failing to reveal a critical limitation of current pertussis vaccines: They do not protect against asymptomatic nasopharyngeal colonization.

A recently developed baboon model has been used to evaluate pertussis vaccine efficacy against whooping cough disease and *B. pertussis* nasopharyngeal colonization and transmission [13]. In this model, whole-cell and acellular vaccines were shown to protect against disease, but not against asymptomatic nasopharyngeal colonization, although the bacterial load upon *B. pertussis* challenge of whole-cell–vaccinated baboons was significantly lower than that of baboons immunized with acellular vaccines [14]. The latter baboons actually were colonized longer than naive baboons. Furthermore, baboons immunized...
with acellular vaccines and subsequently infected with \textit{B. pertussis} could transmit \textit{B. pertussis} to unvaccinated baboons, and when exposed to a colonized host, acellular vaccine–vaccinated baboons were colonized as efficiently as nonvaccinated baboons. In contrast to vaccination, prior infection induced sterilizing immunity in this primate model.

We have developed a live attenuated pertussis vaccine candidate, named BPZE1, for nasal administration. BPZE1 was constructed by the genetic removal or inactivation of 3 \textit{B. pertussis} toxins: pertussis toxin (PT), dermonecrotic toxin, and tracheal cytotoxin [15]. It was shown to be safe in several preclinical models, including highly immunocompromised animals, and to protect mice against \textit{B. pertussis} challenge after a single nasal vaccination (reviewed in [16]). It has now successfully undergone a human phase 1 safety trial and was shown to be safe in human adult males, able to transiently colonize the human respiratory tract and to induce immune responses to several \textit{B. pertussis} antigens in all colonized individuals [17]. Here, we assessed the ability of BPZE1 to protect baboons against both pertussis disease and nasopharyngeal colonization by a high challenge dose of a highly pathogenic clinical \textit{B. pertussis} isolate.

**MATERIALS AND METHODS**

**Ethics Statement**

All animal procedures were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with animal use protocols approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee, ensuring consistency with the principles outlined in the Guide for the Care and Use of Laboratory Animals, the requirements of the US Animal Welfare Act and Regulations, and the US Public Health Service Policy on Humane Care and Use of Laboratory Animals (assurance number A3165-01).

**Bacterial Strains, Culture Media, and Strain Preparation**

Both \textit{B. pertussis} BPZE1 [15] and D420 [18] were grown on freshly prepared Bordet-Gengou (BG) agar plates (Becton Dickinson, Sparks, Maryland) containing 15% defibrinated sheep blood as described previously [19]. After 4 days of growth at 37°C, the bacteria were scraped off the plates, spread onto a fresh BG blood agar plate, and incubated overnight at 37°C. The next morning the bacteria were swabbed up and resuspended into sterile phosphate-buffered saline (PBS). The bacterial suspension was then adjusted to an optical density at 600 nm (OD$_{600}$) of 0.8 to 0.9 to be used as inoculum. After inoculation, the remainder of the suspension was plated out in serial dilutions onto BG blood agar plates and incubated at 37°C for 4 days for bacterial colony-forming unit (CFU) determination.

**Vaccination and Challenge**

Ten healthy, juvenile baboons (\textit{Papio anubis}), approximately 4–5 months of age, were obtained from the Texas Biomedical Research Institute (Southwest National Primate Research Center). Blood samples were taken to screen for \textit{Bordetella bronchiseptica} infection using enzyme-linked immunosorbent assay to detect potential anti–filamentous hemagglutinin (FHA) antibodies. Baboons were sedated by intramuscular injection of 10 mg/kg ketamine and 0.5 mg/kg acepromazine, and BPZE1 or D420 was inoculated as a 1-mL suspension given intratracheally and a 1-mL suspension given intranasally, as described [19]. Four baboons received 10$^8$ CFU BPZE1, and 3 baboons received 10$^{10}$ CFU BPZE1, whereas the remaining 3 baboons were left untreated. Seven weeks later, all baboons were challenged with 1.5 × 10$^{10}$ CFU \textit{B. pertussis} D420.

At the indicated time points after bacterial inoculation, nasopharyngeal washes were performed to determine bacterial colonization as described [19], and blood was drawn to determine white blood cell (WBC) counts by a complete blood count and to measure antibodies against PT, FHA, and pertactin (Prn).

**Antibody Determination**

Pertussis toxin, FHA, and Prn were purchased from List Biologicals and used to coat 96-well plates (Nunc) at 1 µg/well overnight at 4°C in PBS. The plates were then washed 3 times with PBS before blocking for 1 hour at room temperature with 100 µL/well of PBS containing 2% bovine serum albumin (Sigma-Aldrich). Two-fold serial dilutions of 100 µL/well baboon sera in PBS were added and incubated at room temperature for 2 hours. After 6 washes with PBS, 100 µL/well of horseradish peroxidase–conjugated goat antimouse immunoglobulin G (IgG) (BioRad) or antimonkey immunoglobulin A (IgA) (Sigma-Aldrich), diluted 1:10 000 in PBS, was added. After 1 hour of incubation at room temperature, followed by 6 washes with PBS, 100 µL/well of 3,3',5,5'-tetramethylbenzidine (Interchim) was added, and the reaction was stopped after 5 minutes at room temperature by the addition of 50 µL/well of 1 M phosphoric acid. The absorbance was then read at 450 nm.

**Statistical Analysis**

The results were analyzed using the unpaired Student $t$ test and the analysis of variance test (GraphPad Prism software) when appropriate. Differences were considered significant at $P \leq .05$.

**RESULTS**

**BPZE1 Colonization of Baboons**

Four juvenile baboons received 10$^8$ CFU of BPZE1, administered both intranasally and intratracheally. Three others received 10$^{10}$ CFU of BPZE1, and 3 were left untreated. Blood and nasopharyngeal wash samples were collected at different time points, and WBC counts and CFU counts in nasopharyngeal wash samples were determined. In contrast to the nonimmunized animals, BPZE1 could readily be recovered from all baboons vaccinated with 10$^8$ or 10$^{10}$ CFU (Figure 1A). In these
animals, CFU counts declined over time to undetectable levels by day 45 postadministration. Although animals inoculated with \(10^{10}\) CFU seemed to clear BPZE1 faster than those inoculated with \(10^{9}\) CFU, there was no statistical difference between the 2 groups.

Leukocytosis is a hallmark of pertussis, both in humans and in animals [20]. Inoculation of baboons with \(10^9\) or \(10^{10}\) CFU of BPZE1 did not significantly increase WBC counts up to 42 days after administration (Figure 1B). This was expected, as leukocytosis is caused by enzymatically active PT, and BPZE1 produces genetically inactivated PT [15]. Furthermore, no cough episodes were noticed in any BPZE1-inoculated baboon over the entire duration of the experiment, in contrast to previous reports showing that baboons infected with virulent \(B.\) \textit{pertussis} manifest typical pertussis cough [19]. No physical abnormalities were observed after BPZE1 administration, as evidenced by video monitoring, nor was any significant difference observed in red blood cell counts, platelet counts, and hematocrit between treated and nontreated animals (data not shown). These results indicate that BPZE1 is able to transiently colonize the nasopharynx of juvenile baboons without causing pertussis-like symptoms, at least up to \(10^{10}\) CFU.

BPZE1-Induced Serum Antibody Responses Against \(B.\) \textit{pertussis} Antigens

Serum IgG and IgA titers against PT, FHA, and Prn were measured before and after BPZE1 administration. No rise in IgG titers to any of the antigens was seen in the untreated baboons (Figure 2A), whereas IgG to all 3 antigens rose in all animals treated with BPZE1. Antibodies started to rise at 2 weeks after administration and reached a maximum at 4 weeks for PT and Prn, but continued to rise at week 7 for FHA. No significant difference in titer was found between baboons inoculated with \(10^9\) CFU BPZE1 and those inoculated with \(10^{10}\) CFU.

IgA titers against PT, FHA, and Prn also rose in BPZE1-treated baboons. These antibodies also started to appear 2 weeks postadministration and reached maximum levels at 4 or 7 weeks after BPZE1 treatment. Also for IgA titers, no overall statistical differences were observed between the \(10^9\) and the \(10^{10}\) CFU groups. Unexpectedly, 2 nontreated animals also showed detectable, albeit low levels of anti-Prn IgA at 7 weeks, and 1 animal in the \(10^9\) CFU group had high preexisting anti-Prn IgA levels, which were not further increased by BPZE1 administration.

BPZE1-Induced Protection Against a Highly Virulent \(B.\) \textit{pertussis} Challenge

Seven weeks after BPZE1 administration, vaccinated and naïve baboons were infected with approximately \(1.5 \times 10^{10}\) CFU of \(B.\) \textit{pertussis} D420, a highly virulent, recent clinical isolate. Bacterial colonization and blood leukocyte counts were measured over time after challenge. White blood cell counts increased as of 7 days after challenge in nonvaccinated baboons, whereas no significant increase was seen in BPZE1-vaccinated animals at any time point after D420 challenge (Figure 3A). The vaccinated baboons did not cough and had no elevated heart rate or respiration rate. They did not experience any weight loss after challenge and had no other sign of disease. Nonvaccinated baboons appeared sick after D420 challenge, and 1 animal died before the 14-day time point. Therefore, no statistically valid values of WBC counts could be obtained for the nonvaccinated group at 14 days after challenge and beyond. This animal had the highest load of \(B.\) \textit{pertussis} D420 and also the highest leukocyte counts at days 7 and 10 after D420 challenge.

In BPZE1-vaccinated baboons, D420 bacterial counts dropped rapidly after challenge and were undetectable at day 14 postchallenge for all animals. One of the animals vaccinated with \(10^{10}\) CFU BPZE1 had no detectable \(B.\) \textit{pertussis} D420 at any time point, and one \(10^{10}\) CFU-vaccinated baboon had as little as 500 CFU of the challenge strain at day 3 after challenge and no detectable bacteria at day 7 (Figure 3B). A much higher bacterial burden was found in the nonvaccinated animals, in which the D420 challenge was cleared only after 28 days. When
the total *B. pertussis* D420 burden was estimated by measuring the area under the curve, the $10^9$ CFU and the $10^{10}$ CFU BPZE1 vaccine groups had an overall bacterial burden of 0.087% and 0.002%, respectively, compared with the nonvaccinated baboons (Figure 3C).

**Booster Effect of *B. pertussis* Challenge After BPZE1 Priming**

Serum IgG and IgA titers were measured at different time points after *B. pertussis* D420 challenge. Anti-PT IgG titers rose in 2 out of the 3 challenged nonimmunized baboons, starting at 3 weeks after infection to peak at 4 weeks (Figure 4A). One nonimmunized baboon died before the 3-week time point and no antibodies were detectable in this animal. Antibody levels of 1 of the 2 remaining baboons were comparable to those induced by the BPZE1 vaccination, whereas the levels in the second baboon were approximately 3-fold higher. In vaccinated baboons, anti-PT IgG titers rose significantly faster after challenge and reached higher levels than in nonvaccinated animals. Again, there was no significant difference between the 2 BPZE1 dosage groups, and in both groups antibody levels peaked 3 weeks after challenge.

Similar observations were made when anti-FHA IgG was measured. Again, the 2 nonvaccinated baboons showed increasing anti-FHA IgG titers starting 3 weeks after D420 challenge. Average IgG levels were comparable to those obtained after BPZE1 vaccination. Vaccinated baboons again showed a faster and significantly higher IgG response to FHA compared to nonvaccinated animals, with a peak at 3 weeks after challenge and
then a decline. There was again no difference between the 2 vaccinated groups.

Anti-Prn IgG was only modestly induced after D420 challenge of the nonvaccinated baboons, with levels again comparable to those induced by BPZE1 vaccination. However, after challenge of the vaccinated baboons, the anti-Prn IgG responses rose to very high levels, approximately 5- to 10-fold higher than in the nonvaccinated baboons, indicating that efficient priming by BPZE1 had occurred.

Serum anti-PT IgA titers also rose for 1 nonvaccinated animal after challenge, with a peak at 4 weeks after challenge and then a decline (Figure 4B). In the vaccinated animals, anti-PT IgA titers significantly increased at 3 weeks postchallenge and then dropped to prechallenge levels. However, not all animals showed an increase in anti-PT IgA titers after challenge.

Anti-FHA IgA titers rose in the 2 nonvaccinated baboons after D420 challenge to levels higher than those observed after BPZE1 vaccination, and peaked at 3 weeks postchallenge. Earlier IgA responses to FHA were observed in the 2 vaccine groups, with levels comparable to those of the challenged nonvaccinated group.

Finally, anti-Prn IgA were only modestly induced after D420 challenge of nonvaccinated baboons, starting at 4 weeks after challenge, whereas 2- to 5-fold higher levels of anti-Prn were obtained after challenge of vaccinated baboons. Again, these responses occurred earlier in vaccinated than in nonvaccinated animals.

**DISCUSSION**

Previous studies have shown that the baboon model can be used to evaluate the protective effect of pertussis vaccines against both whooping cough disease and *B. pertussis* nasopharyngeal colonization. Whereas both acellular [14] and whole-cell vaccines [13] effectively protect against whooping cough disease, neither prevents nasopharyngeal colonization in these nonhuman primates. In contrast, we show here that BPZE1 protects against both disease and nasopharyngeal colonization by a highly virulent *B. pertussis* strain.
As observed in baboons, immunization with current vaccines also has a minimal effect against *B. pertussis* nasopharyngeal colonization in humans [7, 21]. However, asymptomatic nasopharyngeal colonization leads to transmission [14], which has been recently recognized as playing a major role in the resurgence of pertussis [6].

In this study we used the juvenile baboon model to test the protective efficacy of the live attenuated vaccine candidate BPZE1 against *B. pertussis* colonization with a very high dose of the highly pathogenic recent clinical isolate D420. A single administration of $10^9$ CFU and $10^{10}$ BPZE1 reduced the total nasopharyngeal bacterial burden by 99.913% and 99.998%, respectively, compared with nonvaccinated baboons. In one of the baboons vaccinated with $10^{10}$ CFU BPZE1, no challenge bacteria could be detected at any time point, indicating that BPZE1 had induced sterilizing immunity even against this high dose challenge. A second baboon in the $10^{10}$ BPZE1 CFU group had cleared the infection at 7 days, and the third baboon at 14 days.

Although this study was not designed to directly compare BPZE1 with acellular pertussis vaccines, we nevertheless evaluated the performance of BPZE1 against that of an acellular pertussis vaccine, as published by Warfel et al [14], by using the raw data kindly made available to us by Dr T. Merkel. This comparison showed that a single BPZE1 administration with $10^{10}$ CFU outperformed 3 full human doses of acellular pertussis vaccine.

**Figure 4.** Booster effect of *Bordetella pertussis* D420 challenge in BPZE1-vaccinated baboons. Immunoglobulin G (IgG) (A) and immunoglobulin A (IgA) (B) titers were measured against filamentous hemagglutinin (FHA) (upper panels), pertussis toxin (PT) (middle panels), and pertactin (Prn) (lower panels) at the indicated time points after challenge with *B. pertussis* D420. Each symbol represents an individual animal. Blue, red, and green symbols indicate antibody titers of nonvaccinated animals, baboons vaccinated with $10^9$ colony-forming units (CFU) BPZE1, and baboons vaccinated with $10^{10}$ CFU BPZE1, respectively.
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by 99.8% against total nasopharyngeal bacterial burden using matching protocols. This strong protection against colonization by the D420 strain is remarkable because the challenge strain is a representative of the recent clinical B. pertussis isolates [18], which are very distant from the Tohama I strain, of which BPZE1 is a derivative [15]. Whereas the D420 strain produces serotype 3 fimbriae and harbors the ptxP3 promoter, BPZE1 produces serotype 2 fimbriae and harbors the ptxP1 promoter. It has been suggested that one of the reasons for resurgence of pertussis is the mismatching of vaccine strains with circulating strains [22]; thus, the protection induced by BPZE1 seen here is all the more promising.

BPZE1-vaccinated baboons were also protected against pertussis disease, as evaluated by heart rate, respiration, tissue oxygenation, general well-being, and lack of weight loss, cough, and leukocytosis after challenge, whereas nonvaccinated animals showed increased WBC counts, increased heart and respiration rates, and signs of disease, and 1 of them had to be euthanized due to severe disease burden. Furthermore, BPZE1 vaccination was safe at least up to a dose of 10^{10} CFU, as no significant rise in WBCs was detected, no cough episodes occurred, and no weight loss or physical or behavioral abnormalities were observed.

In addition to demonstrating excellent preclinical safety, BPZE1 was also able to transiently colonize the nasopharynx of baboons. The high-dose vaccine (10^{10} CFU) was cleared somewhat faster than the low-dose vaccine (10^{9} CFU), although not statistically significant, yet there was no difference in serum antibody responses between the 2 dosage groups. The reasons for this clearance differential remain to be determined and may be related to the magnitude of the T-cell responses, mucosal immunity, and/or innate immune effectors induced by the vaccine, which were not investigated in this study. However, the faster clearance of the higher dose observed in this study is in line with the phase 1 clinical trial results in humans, where vaccine clearance rates also accelerated with increasing doses [17]. Whereas 10^{10} CFU of BPZE1 cleared faster than the 10^{9} CFU dose, BPZE1 at 10^{10} CFU appeared to induce better protection against D420 colonization than the 10^{9} CFU BPZE1 dose, suggesting that protection is more related to the initial dose than to the duration of vaccine persistence.

Overall serum IgG and IgA levels to PT, FHA, and Prn were similar in the 2 dosage groups. IgG titers to PT and FHA were high, but anti-Prn antibody titers were modest after BPZE1 vaccination. It is difficult to compare these antibody titers with international standards, as there is no baboon reference serum. Challenge infection with the D420 strain significantly boosted the antibody responses to all 3 antigens. Interestingly, even in the baboon that was vaccinated with 10^{10} CFU BPZE1 and which had no detectable D420 bacterial counts after challenge, the antibody levels to all 3 antigens rose after the challenge at levels comparable to the other vaccinated and challenged animals. Anti-Prn serum IgG levels were even greater in the noncolonized baboon than in the others, indicating that boosting the BPZE1-primed antibody responses by exposure to virulent B. pertussis may not necessarily require colonization by the virulent organism.

Serum IgA titers to the 3 antigens after BPZE1 vaccination were much lower than IgG titers. Again, both dosage groups had similar IgA titers. The reasons for the lower IgA compared to IgG titers are not known. However, this may be due to the sensitivity of the reagents, especially the secondary antibodies. We tested several commercial sources of anti-monkey IgA antibodies, and only 1 gave us measurable titers. Nevertheless, IgA titers to all 3 antigens rose after BPZE1 vaccination and were further boosted by D420 challenge in most animals. Serum IgA to PT, FHA, and Prn are usually minimally or not at all induced after pertussis vaccination in infants [23] but develop after infection [24], most likely via their induction at the respiratory mucosal sites. This suggests that BPZE1 likely induces local IgA production to B. pertussis, which may have contributed to protection against infection. This will be the subject of future studies, which may be important, as this study failed to detect a correlation between serum antibody titers to any of the antigens tested and the level of protection against colonization by B. pertussis D420. However, the numbers of animals were small, and the study was not sufficiently powered to conclude on a lack of serological correlate with protection.

Given mounting evidence that nasopharyngeal colonization with B. pertussis is common, that transmission of B. pertussis by nasopharyngeally colonized individuals plays an important role in the rising incidence of pertussis in recent decades, and that current pertussis vaccines fail to prevent nasopharyngeal B. pertussis colonization, the results of this study provide important evidence of a promising path forward. By not only preventing whooping cough disease, but also substantially reducing nasopharyngeal B. pertussis load, BPZE1 may halt transmission and prove invaluable in reducing the subclinical reservoir of nasopharyngeally colonized individuals. As such, BPZE1 ultimately holds the promise to achieve what has been a challenging and elusive goal of public health for more than a century: effective and long-lasting control of whooping cough.

Notes

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Potential conflicts of interest. C. L. and N. M. hold patents on the BPZE1 vaccine, which is licensed to ILiAD Biotechnologies. M. T., K. S., and K. R. are employees of ILiAD Biotechnologies. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.
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