Mucosal Immunization with Attenuated Salmonella enterica Serovar Typhi Expressing Protective Antigen of Anthrax Toxin (PA83) Primes Monkeys for Accelerated Serum Antibody Responses to Parenteral PA83 Vaccine

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Salmonella enterica serovar Typhi vaccine strain CVD 908-htrA was genetically engineered for stable plasmid-based expression of protective antigen of anthrax toxin (PA83) fused with the export protein ClyA (ClyA-PA83). The priming potential of CVD 908-htrA expressing ClyA-PA83 was assessed in 12 rhesus and 20 cynomolgus macaques that were immunized mucosally (i.e., intranasally) on days 0 and 14. A parenteral booster with purified PA83 plus alum was given to rhesus macaques on days 42 and 225; cynomolgus monkeys received a booster with either PA or licensed anthrax vaccine (BioThrax; Emergent Biosolutions) only one time, 3 months after priming. Monkeys primed with S. Typhi expressing ClyA-PA83 developed high levels of serum toxin-neutralization activity (TNA) antibodies (50% effective dose [ED50], >1.3 × 103), 7 days after receipt of the booster, whereas unprimed controls lacked serum TNA (ED50, 0). In nonhuman primates, the success of this anthrax vaccine strategy based on heterologous mucosal priming followed by a parenteral subunit vaccine booster paves the way for clinical trials.

In 2001, deliberate bioterrorist release of Bacillus anthracis spores resulted in 22 confirmed cases of anthrax, 11 of which were inhalational (5 of these cases were fatal) and 11 of which were cutaneous [1]. The anthrax vaccine licensed by the US Food and Drug Administration, BioThrax (previously known as “anthrax vaccine adsorbed,” or “AVA”; Emergent Biosolutions), derives from a cell-free supernatant of an attenuated, nonencapsulated B. anthracis strain formulated with aluminum adjuvant [2]. Six subcutaneous injections of BioThrax given over 18 months are recommended, followed by annual boosters, to elicit sustained immunity [3]. Serum toxin-neutralizing activity (TNA) antibodies constitute a correlate of protection against inhalational anthrax, on the basis of spore challenges in rabbits and nonhuman primates [4–8]. The primary immunogen of BioThrax that elicits TNA responses is the eukaryotic cell-binding protective antigen (PA) component of anthrax toxin [9–11]. Although placebo-controlled trials and postlicensure surveillance have failed to attribute severe adverse reactions to BioThrax [10–12], the public perception of this vaccine is not positive [13–16]. Absent a tangible bioterror threat, target populations (e.g., laboratory workers, decontaminators, and “first responders”) are reluc-
tant to receive parenteral anthrax vaccine. Efforts are ongoing to produce for US civilians less reactogenic, more immunogenic anthrax vaccines based on highly purified full-length PA83 [8, 17–19] and to stockpile these vaccines. We propose a novel strategy in which mucosally administered, well-tolerated, attenuated Salmonella enterica serovar Typhi live vector vaccine strains expressing PA83 would stimulate strong immunologic priming and memory so that, subsequently, in the face of a bioterror event, primed individuals given a single dose of PA83-based vaccine (BioThrax or purified PA83) would rapidly (i.e., within a few days) attain protective serum TNA levels.

MATERIALS AND METHODS

Culture Conditions and Construction of Vaccine Strains

Plasmids were constructed using standard techniques [20]. CVD 908-htrA, a safe, immunogenic derivative of wild-type strain Ty2, which has deletions in aroC, aroD, and htrA [21], was the live vector. A prokaryotic codon-optimized 2223-bp synthetic gene (GenBank accession no. EU818794) encoding the 735 residues of PA83 [8, 17–19] and to stockpile these vaccines. We propose a novel strategy in which mucosally administered, well-tolerated, attenuated Salmonella enterica serovar Typhi live vector vaccine strains expressing PA83 would stimulate strong immunologic priming and memory so that, subsequently, in the face of a bioterror event, primed individuals given a single dose of PA83-based vaccine (BioThrax or purified PA83) would rapidly (i.e., within a few days) attain protective serum TNA levels.

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**Immunoelectron Microscopy**

Bacterial suspensions were placed on Formvar–carbon–coated nickel grids and incubated with blocking buffer containing 5% BSA-c and 0.1% cold water fish gelatin (Aurion) in PBS, followed by mouse anti-PA and goat anti–mouse IgG conjugated to 10-nm gold particles (Aurion). Grids stained with 1% ammonium molybdate were examined in a JEOL 1200 EX transmission electron microscope.

**Immunizations**

**Rhesus macaques.** Twelve rhesus macaques (*Macaca mulatta*) (body weight, 4.3–5.9 kg) were assembled into 3 groups of 4 animals, with each group having similar weight and sex distributions. The groups were then randomly allocated to be immunized mucosally (i.e., intranasally) on days 0 and 14 with CVD 908-htrA(pSEC91-83), CVD 908-htrA(pPA83), or CVD 908-htrA not carrying an expression plasmid (empty live vector). After being anesthetized with 10 mg/kg ketamine, the animals were placed in a sitting position, and 50 μL of vaccine suspension containing 4.7 × 109–1.1 × 1010 cfu (hereafter referred to as “≈5 × 109 cfu”) in PBS were instilled into the nares (25 μL/nare). On day 42, the monkeys were given an intramuscular booster with purified PA83 (42.5 μg) adsorbed to 0.75 mg of alum (VaxGen; developmental lot 8). A second PA83 booster (VaxGen; developmental lot 041906) was given to all monkeys 6 months later (on day 225).

**Cynomolgus macaques.** Twenty cynomolgus macaques (*Macaca fascicularis*) (body weight, 2.5–4.0 kg) were assembled by weight and sex into 2 similar groups of 12 and 8 animals. On days 0 and 14, animals in the group of 12 were randomly allocated to receive intranasal immunization with 3.2 × 108–8.0 × 109 cfu (hereafter referred to as “≈−5 × 109 cfu”) of CVD 908-htrA(pSEC91-83), whereas the group of 8 received CVD 908-htrA carrying pSEC91 without PA genes (empty expression plasmid). Three months after mucosal priming (on day 100), one-half of the animals in each group were randomly allocated to receive an intramuscular booster of 85 μg of PA83 adsorbed to 0.75 mg of alum (VaxGen; developmental lot VXG-0827), and the other half were allocated to receive 0.5 mL of BioThrax (Emergent Biosolutions; lot FAV119) subcutaneously. Blood samples were collected before and after immunization.

The studies were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

**Antibody Responses**

Serum TNA antibodies were measured using the assay of Quinn and colleagues [8, 23, 24]. Titers were calculated using an endpoint algorithm and were reported as the reciprocal of a serum dilution that resulted in 50% neutralization of toxin-mediated cytotoxicity (or a “50% effective dose” [ED50]). The serum level of IgG to PA was measured by ELISA. Plates were coated with PA83 (List Biological) at 2 μg/mL in PBS and were blocked with 10% dry milk in PBS. Duplicate samples were tested in serial dilutions. HRP-labeled anti–monkey IgG (KPL) and anti-human IgG1–4 (The Binding Site) were used as conjugates, followed by tetramethylbenzidine (TMB) substrate (KPL). Anti-PA IgG titers were calculated by interpolation of regression-corrected absorbance values for experimental samples into a standard curve, and they were expressed as micrograms per milliliter. Through linear regression analysis, anti-PA IgG subclass titers were calculated as the inverse of the dilution that produces an absorbance value of 0.2 above the blank. Respiratory secretions were not collected to measure secretory IgA anti-PA.

**Statistical Analysis**

Antibody titers in selected groups were compared using the Mann-Whitney U test. P < .05 was considered to be statistically significant. No adjustment was made for multiple comparisons, because, with the small numbers of animals per group, we elected not to control the overall type I error rate at <.05. Statistical analyses, including linear regression, were performed using Sigma Stat software (version 3.0; SPSS).

**RESULTS**

**Expression of PA83 in S. Typhi.** Whole-cell lysates of CVD 908-htrA(pSEC91-83) and CVD 908-htrA(pPA83) expressing, respectively, PA83 fused to the export protein ClyA or unfused PA83 (figure 1A) were separated on SDS-polyacrylamide gels. After staining with Coomassie brilliant blue (figure 1B, top), protein bands approximating the expected molecular masses for ClyA-PA83 and unfused PA83 (116.6 kDa and 83.0 kDa, respectively) were observed. Western blot analysis with anti-PA83 polyclonal antiserum also detected proteins of ~117 and 83 kDa (figure 1B, bottom). Smaller protein species were detected in these lysates, likely as a result of proteolytic cleavage of PA83.

**Immunoelectron microscopy.** Immune labeling with gold particles observed by electron microscopy confirmed that ClyA-PA83 fusions were exported out of the live vector cytoplasm. CVD 908-htrA(pSEC91-83) exhibited a high density of gold particles on the cell surface (figure 2A). In contrast, no gold particles were seen on the surface of (1) CVD 908-htrA expressing cytoplasmic PA83 (figure 2B) or empty CVD 908-htrA live vector (figure 2C) incubated with antibodies specific for PA, or (2) CVD 908-htrA(pSEC91-83) incubated with nonimmune mouse serum samples (figure 2D).
with purified PA (median titer, 1701 and 225, respectively, vs. 0 in unprimed controls; \( P = .029 \)). TNA responses peaked on day 56, which was 2 weeks after receipt of the booster, with median titers of 6755 and 2255 ED\(_{50}\), respectively, noted for the ClyA-PA83 and unfused PA83 groups. At all time points from day 49 through 239, the monkeys primed with PA (with ClyA and cytoplasmic data pooled) had titers that were significantly higher than those of the unprimed monkeys. Beyond the first month after receipt of the booster, the titers decreased rather slowly but remained elevated for >6 months. TNA titers also decreased in the unprimed animals, remaining only slightly above the detection limit. TNA titers in monkeys primed with S. Typhi expressing CVD 908-htrA (pSEC91-83) were 2- to 5-fold higher than those noted in animals primed with unfused PA83; on days 56 and 189, the differences in the TNA titers between these 2 groups approached statistical significance (\( P = .057 \)).

Figure 2 shows the kinetics of the serum IgG anti-PA responses. With the use of this ELISA, modest anti-PA titers were observed after priming with S. Typhi expressing either form of PA, with 50% of animals experiencing seroconversion after receiving 2 doses of live vector. A rapid, strong anamnestic serum anti-PA response was observed in these animals on day 49, which was 7 days after a parenteral booster with purified PA was received on day 42. In contrast, no responses were detected on day 49 in unprimed monkeys who had received S. Typhi not encoding PA83. Median concentrations of anti-PA IgG in macaques primed with ClyA-PA83, unfused PA83, and empty live vector were 604, 226, and 0.41 \( \mu \)g/mL, respectively, at day 49. The peak responses in monkeys primed with live vectors expressing PA83 appeared on day 56, and they were >10-fold higher than the peak responses of unprimed animals (\( P = .008 \)), whose peak titers occurred 2 weeks later on day 70. At all time points, antibody levels were ~2-fold higher in animals primed with CVD 908-htrA expressing ClyA-PA83 than in animals primed with strains expressing unfused PA83; however,
only at day 99 was the difference statistically significant ($P = .029$).

The rhesus macaques received a second parenteral immunization with PA83 on day 225 (6 months after receipt of the first parenteral booster). The TNA and IgG anti-PA responses were similar to those observed after the receipt of a first parenteral booster with PA (figure 3A and 3B). In animals primed with ClyA-PA83, TNA titers remained elevated long after receipt of a booster, with a median titer of 5945 ED$_{50}$ noted on day 314 (the last time point measured); serum TNA levels decreased more precipitously in animals primed with unfused PA83 (median titer, 1119 ED$_{50}$) ($P = .029$, compared with animals primed with ClyA-PA83) and unprimed animals (median titer, 768 ED$_{50}$).

**Antibody responses to PA83 in cynomolgus macaques.** The second experiment, which was performed in cynomolgus macaques, had 3 objectives: (1) to confirm the reproducibility of the mucosal prime-parenteral booster immunization strategy by use of a different nonhuman primate model; (2) to test the capacity of this vaccine to prime memory B cells that would respond with a potent anamnestic humoral response to a more-delayed (3 months after priming) PA83 booster; and (3) to compare the relative potency of different parenteral boosters (purified PA83 vs. BioThrax) to elicit anamnestic serum TNA responses.

Two cohorts of cynomolgus macaques containing 12 and 8 monkeys each were primed with CVD 908-htrA(pSEC91-83) or CVD 908-htrA carrying empty expression plasmid pSEC91, respectively. Three months later, one-half of the animals in each cohort received a parenteral booster with purified PA83, and the other half received a booster with BioThrax. The kinetics of the TNA and IgG anti-PA responses in the cynomolgus macaques (figure 4A and 4B) were remarkably similar to those seen in rhesus macaques. After mucosal priming with CVD 908-htrA(pSEC91-83), 83% of the monkeys manifested significant increases in serum IgG anti-PA83, whereas unprimed animals immunized with CVD 908-htrA(pSEC91) failed to show detectable IgG anti-PA83.

Cynomolgus macaques primed with CVD 908-htrA(pSEC91-83) exhibited a rapid and strong antibody recall response on day 107, which was 7 days after receipt of a booster (on day 100) with either PA83 or BioThrax, despite the longer interval between mucosal priming and receipt of a parenteral booster. The serum TNA titers in cynomolgus monkeys measured on day 107, one week after receipt of a booster with either PA83 or BioThrax, showed that none of the unprimed monkeys who received CVD 908-htrA(pSEC91) had TNA antibodies. In contrast, 11 of 12 monkeys primed with CVD 908-htrA(pSEC91-83) demonstrated TNA antibodies after receiving a booster with PA83 (6 of 6 monkeys demonstrated a response; median titer, 2255 ED$_{50}$; $P = .01$, compared with unprimed monkeys) or BioThrax (5 of 6 monkeys demonstrated a response; median titer, 519 ED$_{50}$; $P = .038$). There was no statistically significant difference in the TNA response in monkeys given VaxGen PA83 versus those given BioThrax at any
time point. One monkey primed with CVD 908-htrA(pSEC91-83) failed to respond to the BioThrax booster, despite experiencing seroconversion for anti-PA after live vector priming. This animal received immunization identical to that received by other animals in this group, and there were no records of health-related or technical issues that could account for the lack of response in this animal.

**IgG subclass profiles.** The IgG subclass anti-PA profiles observed in rhesus and cynomolgus monkeys after receipt of the parenteral booster with vaccine containing PA83 are shown in figures 5 and 6. There were no significant increases in any anti-PA IgG subclasses after priming alone, but all animals exhibited increases in anti-PA of all subclasses after the parenteral booster, peaking 14 days later. Higher IgG1 and IgG2 levels were observed in monkeys primed with CVD 908-htrA expressing PA83, compared with animals who received empty live vector; increases in IgG3 and IgG4 anti-PA were also observed, albeit at much lower levels (figures 5A and 6A). In both experiments, an excellent correlation was found between IgG1 and IgG2 titers and TNA, with $r = 0.94$ and $r = 0.96$, respectively, noted for rhesus macaques ($P < .001$).
and **r**/H11005 0.92 and **r**/H11005 0.93, respectively, noted for cynomolgus macaques (**P*/H11021 .001) (figure 6).

**DISCUSSION**

Devising a strategy to protect potentially high-risk subgroups of the US civilian population in the event of a deliberate bioterrorist release of anthrax spores, such as that which occurred in 2001, poses practical dilemmas. Because the only licensed anthrax vaccine requires multiple spaced doses and many weeks to achieve protective antibody levels, this argues for prophylactic immunization for such groups. However, poor public perception of the safety of the currently licensed parenteral anthrax vaccine renders many members of high-risk groups reluctant to undergo preincident immunophylaxis [14]. If another spore release

**Figure 6.** Anti–protective antigen (PA) IgG subclass profile in cynomolgus macaques primed with *Salmonella enterica* serovar Typhi expressing ClyA-PA83 and given a booster with either PA of anthrax toxin (PA83) or BioThrax (Emergent Biosolutions) according to the immunization schedule described in figure 4. A, Kinetics of IgG1–4 anti-PA responses. Data points represent denote median antibody concentrations, with bars denoting 95% confidence intervals. Statistical tests (the Mann-Whitney **U** test) compared titers of individual animals in different groups at the various time points shown, and statistical significance designations are defined as follows: *P* = .01 for CVD 908-htrA(pSEC91-83)–primed animals given a booster with either vaccine containing PA vs. unprimed animals; **1P** = .01, for CVD 908-htrA(pSEC91-83)–primed animals given a booster with VaxGen PA vs. unprimed animals, and **P** = .038, for CVD 908-htrA(pSEC91-83)–primed animals given a booster with BioThrax PA vs. unprimed animals; † **P** = .01, for CVD 908-htrA(pSEC91-83)–primed animals given a booster with VaxGen PA vs. unprimed animals; ‡ **P** = .038, for CVD 908-htrA(pSEC91-83)–primed animals given a booster with BioThrax PA vs. unprimed animals. **B,** Linear regression analysis of IgG1 and IgG2 levels (x-axis) and toxin-neutralization activity (y-axis) (**P*/H11021 .001). Data points denote titers of individual animals measured on day 113 (peak responses).
occurs and the risk of anthrax becomes tangible, then attitudes would change, and members of these groups would likely seek vaccination. The challenge then would be to confer protection rapidly. Disappointingly, heretofore in clinical trials, the new parenteral PA vaccines have not diminished the lag until protective levels of TNA appear [18, 19].

In a novel approach to resolve this dilemma, we propose a “hybrid” vaccination strategy in which a well-tolerated, attenuated S. Typhi live vector strain administered mucosally before the incident is used to prime the immune system and elicit strong immunologic memory such that, subsequently, at the time of an incident, parenteral administration of a single dose of PA-based vaccine (BioThrax or purified PA) will rapidly (i.e., within a few days) elicit protective levels of serum TNA. This strategy assumes that the reluctance of high-risk groups to receive a parenteral anthrax vaccine for priming discourages that option and that a well-tolerated oral priming vaccine will achieve much higher compliance [25, 26]. In the present study, we provide highly encouraging, preliminary evidence to support the feasibility of this approach.

S. Typhi strain CVD 908-htrA was chosen as the live vector because it has been well tolerated and immunogenic in phase 2 clinical trials, both as a live oral typhoid vaccine and as a live vector [21, 27]. Monkeys were immunized intranasally rather than orally, which is the natural route by which live typhoid vaccines are given [21, 28], because the narrow host restriction of S. Typhi makes it difficult to infect experimental animals orally. This caveat aside, these experiments in monkeys provide an unequivocal proof of principle of the effectiveness of mucosal priming. Monkeys primed mucosally with S. Typhi expressing PA83 exhibited >100-fold increases in serum TNA and IgG anti-PA antibodies a mere 7 days after receipt of a single parenteral booster with PA-based vaccine. In contrast, no TNA response (and only a negligible IgG anti-PA response) was observed 7 days after parenteral administration of PA vaccine to unprimed animals.

Additional observations attest to the robustness of mucosal priming with S. Typhi live vector expressing PA83: (1) the S. Typhi live vectors functioned in 2 species of macaques; (2) strong, rapid anamnestic responses ensued whether the parenteral booster was administered with BioThrax or purified PA; (3) mucosally primed rhesus macaques maintained elevated serum TNA antibody titers for multiple months following an initial parenteral boost with PA83, with only a slow decrease noted (figure 3); and (4) in mucosally primed animals, the responses to a parenteral booster with vaccine containing PA83 were robust after a lag of 3 months as after 1 month. The novel observation that monkeys can be successfully immunized intranasally with S. Typhi, thereby overcoming the notorious host restriction of that serovar when it is given orally, can accelerate S. Typhi live vector vaccinology. Indeed, these encouraging data pave the way for clinical trials of this strategy in humans who would receive oral priming with S. Typhi vector. Under the assumption that initial human clinical trials corroborate the results of the monkey studies, subsequent clinical trials would establish the effectiveness of a single oral priming dose and would examine much longer lag times (e.g., 6, 12, and 24 months) before administration of the single parenteral PA booster. Human trials will also aim to detect serum TNA at time points earlier than 7 days after receipt of the booster.

Immunoelectron microscopy studies confirmed that ClyA-PA83 fusions were exported to the outer surface of CVD 908-htrA(pSEC91-83) (figure 2A), which is an important observation because foreign antigens exported to the surface of or external to Salmonella live vectors are generally more immunogenic than are the same antigens expressed within the cytoplasm or periplasm [22, 29–31]. This is true for PA83 [32] and domain 4 of PA [22]. The ClyA export system appears to be particularly effective. Mice orally immunized with Salmonella enterica serovar Typhimurium expressing ClyA-PA83 [32] were protected against B. anthracis aerosol spore challenge, whereas similar constructs involving fusion of PA83 to an Escherichia coli hemolysin A plasmid-based secretion system failed to provide protection [32]. The experiments in rhesus macaques reported herein shed some light on the relative effectiveness of the ClyA-PA83 export system when used in a heterologous mucosal prime-parenteral booster strategy. Rhesus macaques primed with S. Typhi expressing ClyA-PA83 fusions exhibited 2- to 5-fold higher serum TNA antibody titers at all time points after administration of a dose of parenteral purified PA (figure 3) than did monkeys primed with unfused PA83; however, the differences were not significant.

The antibody responses that developed after receipt of the parenteral booster were long-lasting and could be rapidly increased further with a second booster with PA83 given ~6 months after the initial parenteral booster was received (figure 3). This finding suggests the presence of live vector–primed memory B cells that are ready to increase antibody production after subsequent recall immunizations. Similar kinetics of serum IgG and TNA responses were reported by Pittman et al. [33] in humans immunized with multiple parenteral doses of AVA.

Because of the limited number of monkeys available, we could not include a comparator group that was primed parenterally with PA-based vaccine before receipt of a booster with an additional parenteral dose. Nevertheless, the lack of serologic data from such a control group is not deemed to be a significant shortcoming, for several reasons. First, the rationale for pursuing the mucosal priming strategy is that preincident parenteral priming simply does not appear to be a viable option. Second, our heterologous prime-boost immunization strategy produced peak serum TNA levels in rhesus monkeys (median titer, 6755 ED₅₀) that were very similar to those reported by Williamson et al. [8], who immunized rhesus macaques parenterally with 2 doses of PA83 (50 µg/dose) 4 weeks apart. These monkeys ex-
hhibited serum TNA antibody levels of ~5000 ED₅₀ 6 weeks after receipt of the booster and were fully protected against aerosol challenge with the spores of B. anthracis Ames strain. Third, as seen in figure 3, for rhesus macaque controls receiving empty S. Typhi live vector on day 0 and intramuscular booster doses of PA on days 42 and 225, the median serum TNA antibody titer on day 239 (14 days after receipt of the booster) is similar to that noted on day 56 for macaques primed mucosally with the ClyA-PA S. Typhi construct and given an intramuscularly administered booster with PA on day 42.

Monkeys primed with S. Typhi expressing PA83 mainly exhibited increases in IgG1 and IgG2 subclasses, which correlated with serum TNA. IgG1 binds complement and all 3 Fcγ cellular receptors, mediates bactericidal and opsonophagocytic activity [34], and is associated with toxin neutralization in humans. The contribution of IgG2, which does not bind cell receptors, is less clear. Williamson et al. [8] reported increases in IgG1 and IgG2 but not IgG3 and IgG4 anthrax antibodies in rhesus macaques immunized with PA83-alum. IgG1, IgG2, and low levels of IgG3 anthrax antibodies were also described in humans who received multiple doses of AVA, as well as in patients with inhalational anthrax from the 2001 bioterrorist spore disseminations [35]. Appreciable levels of IgG3 and IgG4 were produced only when mucosal priming with S. Typhi expressing PA83 preceded the parenteral PA83 booster (figures 5A and 6A), suggesting that live vector priming may modulate responses to the parenteral booster. The broader response elicited by live vector priming before parenteral administration of PA may enhance protection.

The results of our monkey studies support the feasibility of a modified preexposure vaccination strategy wherein a well-tolerated S. Typhi live vector anthrax vaccine is administered to the high-risk population to prime them immunologically to mount rapid, protective serum TNA responses within a few days of receiving a single dose of PA-based vaccine. Such an approach would diminish the total number of doses needed to achieve protection in priority groups (diminishing pressure on stockpiles of vaccine) and could create protected cohorts within a few days of administration of the booster.

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