MAJOR ARTICLE

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

James E. Galen,[1,2] Magaly Chinchilla,[1,2] Marcela F. Pasetti,[1,3] Jin Yuan Wang,[1,2] Licheng Zhao,[1,2] Ivonne Arciniega-Martinez,[1,3] David J. Silverman,[4] and Myron M. Levine[1,2,3]

[1]Center for Vaccine Development, [2]Division of Geographic Medicine, Department of Medicine, [3]Division of Infectious Diseases and Tropical Pediatrics, Department of Pediatrics, and [4]Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore

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 [ED_{50}], $>1.3 \times 10^3$), 7 days after receipt of the booster, whereas unprimed controls lacked serum TNA (ED_{50}, 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-

Received 27 June 2008; accepted 22 August 2008; electronically published 19 December 2008.

Potential conflicts of interest: J.E.G. holds patents on the plasmid maintenance system and the ClyA antigen export system. M.M.L. was a member of the Board of Directors of VaxGen while much of this work was ongoing. All other authors: no conflicts.

Presented in part: J.E.G. presented interim data at the Regional Center of Excellence annual meetings in Galveston, Texas, 13–15 March 2005; New York, New York, 26–28 March 2006; St. Louis, Missouri, 15–17 April 2007; and Chicago, Illinois, 6–8 April 2008; as well as at the Annual Conference on Vaccine Research meetings of the National Foundation for Infectious Diseases, Baltimore, Maryland, 8–10 May 2006 (abstract S18) and 30 April–2 May 2007 (abstract S20). M.C. presented interim data at the 106th General Meeting of the American Society for Microbiology, Orlando, Florida, 21–25 May 2006 (abstract E-021), and the annual meeting of the Federation of Clinical Immunology Societies, Boston, Massachusetts, 5–9 June 2008 (abstract Su.38).

Financial support: Mid-Atlantic Regional Center for Excellence for Biodefense and Emerging Infectious Research (cooperative agreement grant U54 AI57168 [to M.M.L.]); National Institutes of Health (NIH; grants R01 AI29471 [to M.M.L.] and R01 AI065760 [to M.F.P.]); National Institute of Allergy and Infectious Diseases, NIH (research contract NO1 AI25461 [to M.M.L.]).

Reprints or correspondence: Dr. Myron M. Levine, Center for Vaccine Development, University of Maryland School of Medicine, 685 W. Baltimore St., Baltimore MD 21201 (mlevine@medicine.umaryland.edu).

The Journal of Infectious Diseases 2009; 199:326–35

0022-1899/2009/19903-0006$15.00
DOI: 10.1086/596066

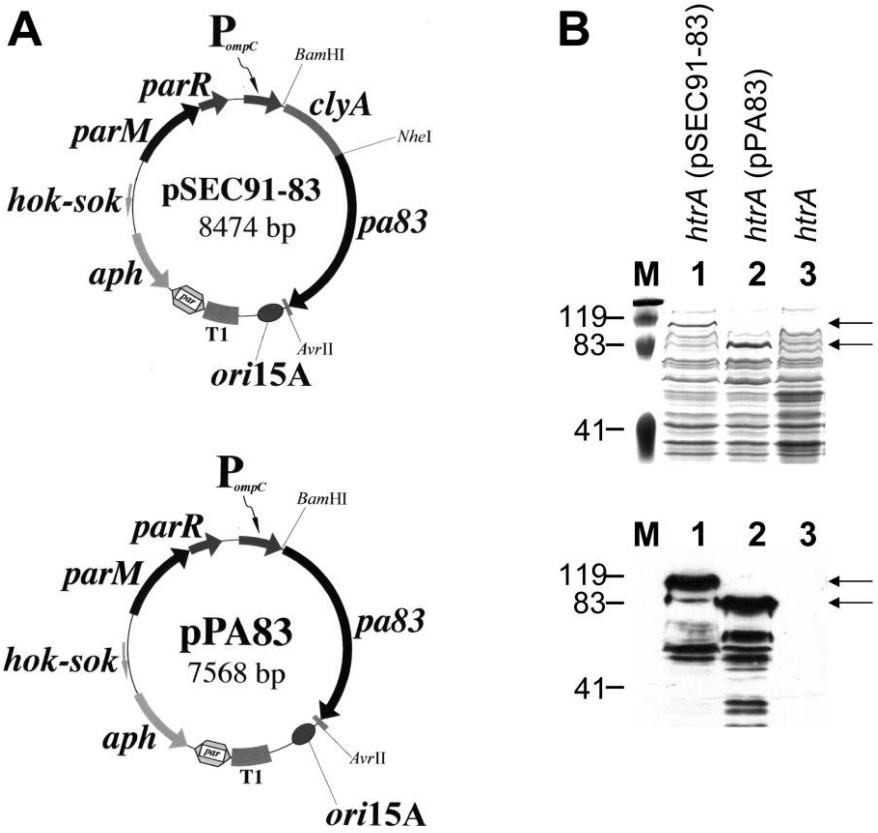

Figure 1. Plasmid-based expression of protective antigen (PA) of anthrax toxin (PA83) in CVD 908-*htrA* live vectors. *A,* Genetic maps of the isogenic expression plasmids encoding ClyA-PA83 and PA83 immunogens. *B, Bacillus anthracis* PA expression in vaccine constructs. Coomassie brilliant blue-stained SDS-polyacrylamide gel *(top)* and Western immunoblot *(bottom)* analysis of whole cell lysates from either CVD 908-*htrA*(pSEC91-83) *(lane 1),* CVD 908-*htrA*(pPA83) *(lane 2),* or empty CVD 908-*htrA* live vector as a control *(lane 3).* Immunoblot membranes were probed with polyclonal antiserum raised against PA83, as described in Materials and Methods. Arrows denote the expected molecular masses for ClyA-PA83 (116.6 kDa) and PA83 (83.0 kDa), respectively. *aph,* gene encoding the aminoglycoside 3'-phosphotransferase conferring resistance to kanamycin; *clyA,* gene encoding cytolysin A (ClyA) from *Salmonella enterica* serovar Typhi; *hok-sok,* postsegregational killing locus from the multiple antibiotic resistance R-plasmid pR1; *ori*15A, origin of replication from pACYC184, providing an expected expression plasmid copy number of ~15 copies/chromosomal equivalent; *pa83,* gene encoding full-length *B. anthracis* PA, codon-optimized for expression in Typhi; *parM* and *parR,* components of the *parA* active partitioning system from pR1; P_{ompC}, modified, osmotically controlled *ompC* promoter from *Escherichia coli;* T1, transcriptional terminator from the *rrnB* rRNA operon of *E. coli.*

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 was chemically synthesized as a *Nhe*I-*Avr*II cassette by GenScript, and it encoded a protein with an expected molecular mass of 83.0 kDa. This cassette was inserted in frame into the unique *Nhe*I site at the carboxyl terminus of the antigen export protein ClyA, encoded by genetically stabilized expression plasmid pSEC91 [22], creating pSEC91-83 (figure 1*A*). Insertion of the identical cassette into pSEC91 cleaved with *Spe*I and *Nhe*I removed *clyA* to create isogenic construct pPA83 expressing cytoplasmic PA83 (figure 1*A*). Both constructs were confirmed by DNA sequence analysis. After electroporation of these plasmids into CVD 908-*htrA,* expression of ClyA-PA83 and unfused PA83 was examined by Western immunoblotting [22] performed with the use of goat anti-PA IgG (List Biological) and horseradish peroxidase (HRP)–labeled rabbit anti–goat IgG (Kirkegaard and Perry Laboratories [KPL]).

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 skin 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 4.7×10^9–1.1×10^{10} cfu (hereafter referred to as "$\sim 5 \times 10^9$ 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 3.2×10^9–8.0×10^9 cfu (hereafter referred to as "$\sim 5 \times 10^9$ 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" [ED_{50}]). 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 1*A*) were separated on SDS-polyacrylamide gels. After staining with Coomassie brilliant blue (figure 1*B, 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 $\sim$117 and 83 kDa (figure 1*B, 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 2*A*). In contrast, no gold particles were seen on the surface of (1) CVD 908-*htrA* expressing cytoplasmic PA83 (figure 2*B*) 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 2*D*).

Antibody responses to PA83 in rhesus macaques. Figure 3*A* displays serum TNA responses. No monkeys manifested increases in serum TNA antibodies after priming alone. Nevertheless, the animals primed with *S.* Typhi expressing PA83 (ClyA-PA83 or unfused) developed TNA responses by 7 days (the earliest time point tested) after receipt of a parenteral booster

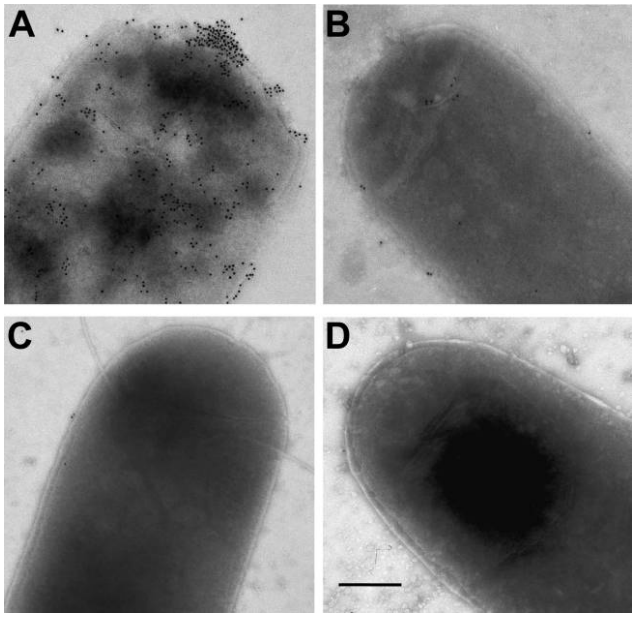

Figure 2. Expression of protective antigen (PA) on the surface of *Salmonella enterica* serovar Typhi CVD 908-*htrA*(pSEC91-83) and CVD 908-*htrA*(pPA83), as determined by immunogold staining. Immunoelectron micrographs of *S.* Typhi CVD 908-*htrA*(pSEC91-83) *(A), S.* Typhi CVD 908-*htrA*(pPA83) *(B),* and *S.* Typhi CVD 908-*htrA (C)* incubated with mouse PA-specific antibodies and gold-labeled anti–mouse antibody. *D,* CVD 908-*htrA*(pSEC91-83) incubated with nonimmune serum and gold-labeled anti–mouse antibody. The bar denotes 0.25 μm.

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 ClyA-PA83 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 3*B* 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 μg/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,

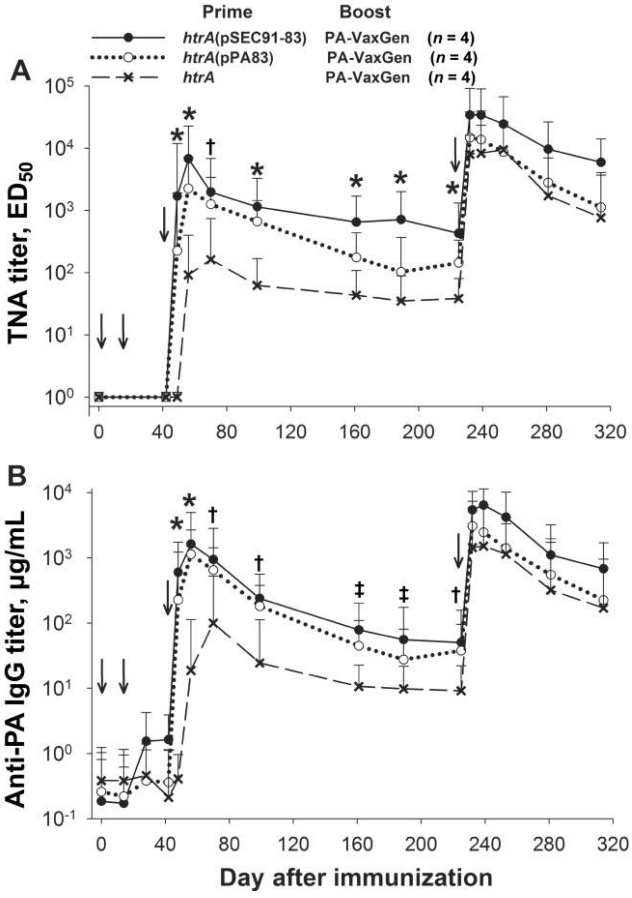

Figure 3. Toxin-neutralization activity (TNA) titers *(A)* and anti–protective antigen (PA) IgG titers *(B)* in rhesus macaques primed intranasally with ~5×10^9 cfu of either CVD 908-*htrA*(pSEC91-83), CVD 908-*htrA*(pPA83), or CVD 908-*htrA* empty live vector on days 0 and 14 and then, on day 42 (and again on day 225), intramuscularly administered a booster with PA83 adsorbed to alum (VaxGen). Arrows denote the immunizations. Data points denote median titers of TNA or median concentrations of IgG anti-PA, with bars denoting 95% confidence intervals. Statistical tests (the Mann-Whitney *U* test) compared the titers of individual animals in the different groups with respect to the various time points, and statistical significance designations are defined as follows: *$P = .029$, for animals primed with *Salmonella enterica* serovar Typhi CVD 908-*htrA* carrying either (pSEC91-83) or (pPA83) vs. unprimed control animals that received *S.* Typhi CVD 908-*htrA* without plasmid; †$P = .029$, for CVD 908-*htrA*(pSEC91-83) vs. control, and $P = .057$, for CVD 908-*htrA*(pPA83) vs. control; ‡$P = .029$, for CVD 908-*htrA*(pSEC91-83) vs. control, and $P = .20$, for CVD 908-*htrA*(pPA83) vs. control.

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 3*A* and 3*B*). 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 4*A* and 4*B*) 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

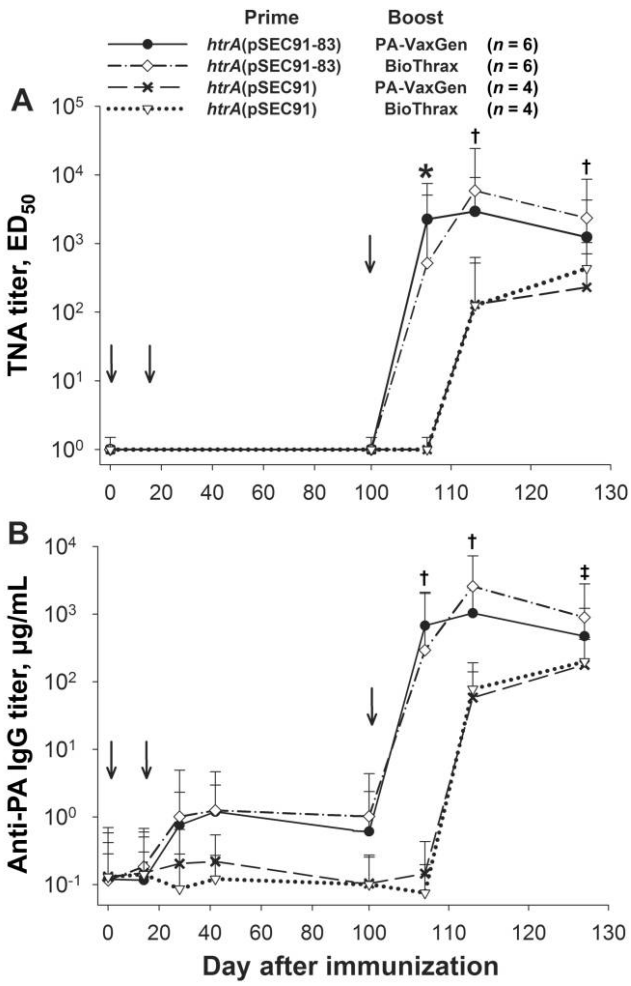

Figure 4. Toxin-neutralization activity (TNA) titers *(A)* and anti–protective antigen (PA) IgG titers *(B)* in cynomolgus macaques that were primed intranasally with $\sim 5 \times 10^9$ cfu of CVD 908-*htrA*(pSEC91-83) or CVD 908-*htrA*(pSEC91) (empty expression plasmid) on days 0 and 14 and were given a booster with either PA83 adsorbed to alum (VaxGen) given intramuscularly or BioThrax (Emergent Biosolutions) given subcutaneously on day 100. Arrows denote each immunization. Data points denote median titers of TNA or median concentrations of IgG anti-PA, with bars denoting 95% confidence intervals. Statistical tests (the Mann-Whitney *U* test) compared the titers of individual animals in the different groups at the various time points shown. *A,* Statistical significance designations, defined as follows: $*P = .01$, 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 vs. control animals; $\dagger P = .01$, for CVD 908-*htrA*(pSEC91-83)–primed animals given a booster with VaxGen PA vs. unprimed animals, and $P = .114$, for CVD 908-*htrA*(pSEC91-83)–primed animals given a booster with BioThrax vs. control animals. *B,* Statistical significance designations are defined as follows: $\dagger P = .01$, for CVD 908-*htrA*(pSEC91-83)–primed animals given a booster with VaxGen PA vs. unprimed animals, and $P = .114$, for CVD 908-*htrA*(pSEC91-83)–primed animals given a booster with BioThrax vs. control animals; $\ddagger P = .038$, for CVD 908-*htrA*(pSEC91-83)–primed animals given a booster with VaxGen PA vs. unprimed animals, and $P = .114$, for CVD 908-*htrA*(pSEC91-83)–primed animals given a booster with BioThrax vs. control animals.

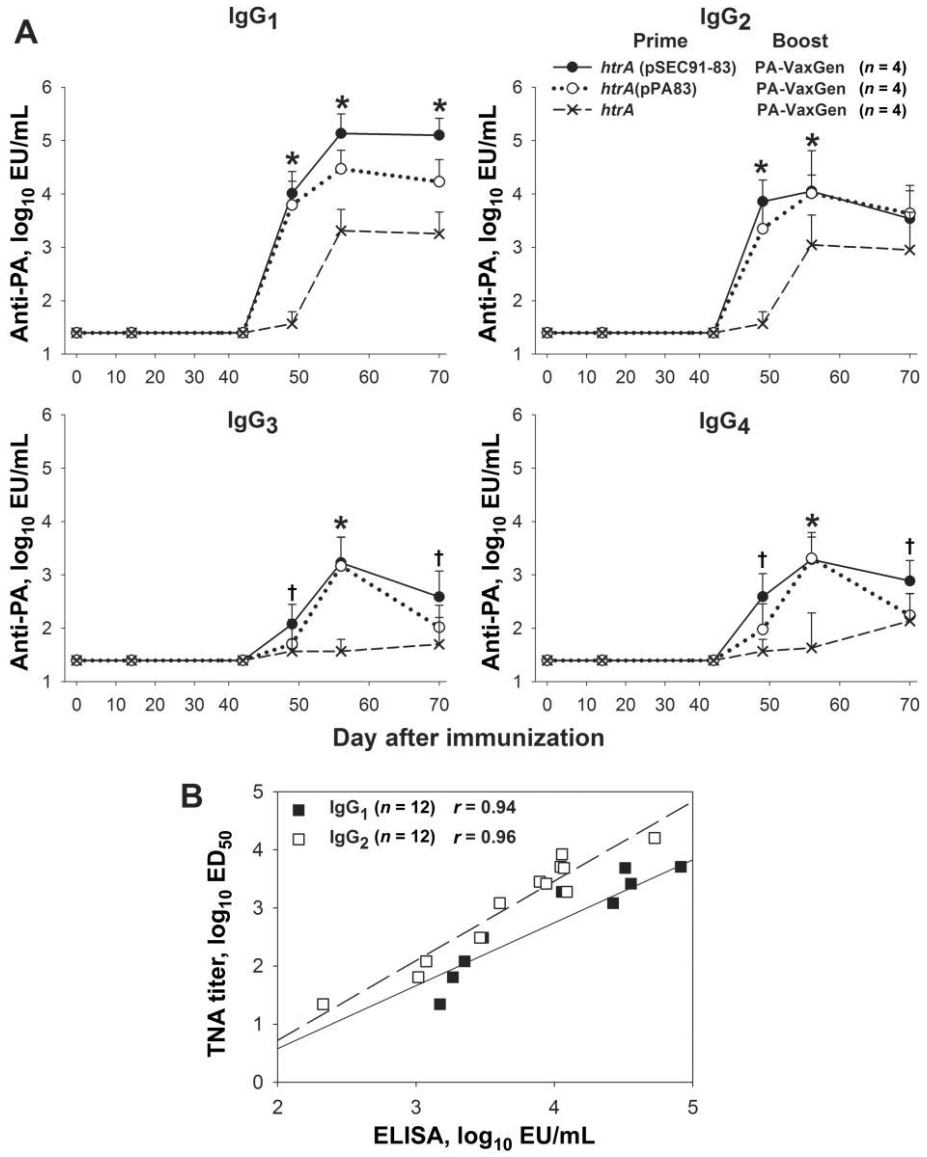

Figure 5. Anti–protective antigen (PA) IgG subclass profile in rhesus macaques primed with *Salmonella enterica* serovar Typhi expressing PA of anthrax toxin (PA83) and given a booster with PA83-alum according to the immunization schedule shown in figure 3. *A,* Kinetics of IgG1–4 anti-PA responses. Data points denote median antibody concentrations, with bars denoting 95% confidence intervals. Statistical tests (the Mann-Whitney *U* test) compared titers of individual animals in the different groups at the various time points shown. Statistical significance designations are defined as follows: $*P = .029$, for animals primed with *S.* Typhi CVD 908-*htrA* carrying either (pSEC91-83) or (pPA83) vs. unprimed control animals that received *S.* Typhi CVD 908-*htrA* without plasmid; $\dagger P = .029$, for CVD 908-*htrA*(pSEC91-83) vs. control. *B,* Linear regression analysis of IgG1 and IgG2 levels (*x*-axis) and toxin-neutralization activity (*y*-axis) ($P < .001$). Data points denote titers of individual animals on day 56 (peak responses).

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 5*A* and 6*A*). 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 ($P < .001$)

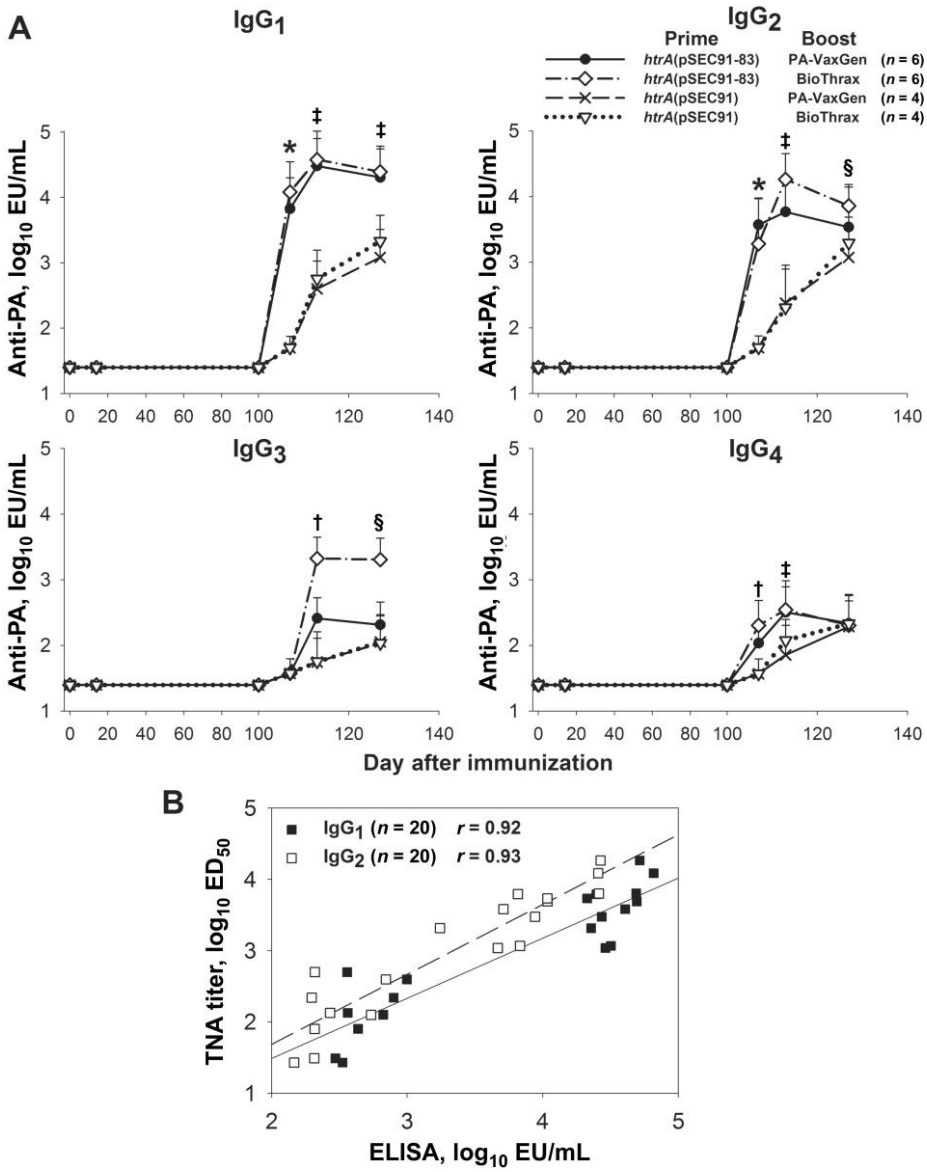

Figure 6. Anti–protective antigen (PA) IgG subclass profile in cynomolgus macaques primed with *Salmonella enterica* serovar Typhi expressing ClyA-PA83 and given a booster with either PA of anthrax toxin (PA83) or BioThrax (Emergent Biosolutions) according to the immunization schedule described in figure 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; †*P* = .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 VaxGen PA vs. unprimed animals. *B,* Linear regression analysis of IgG1 and IgG2 levels (*x*-axis) and toxin-neutralization activity (*y*-axis) (*P* < .001). Data points denote titers of individual animals measured on day 113 (peak responses).

(figure 5*B*) and $r = 0.92$ and $r = 0.93$, respectively, noted for cynomolgus macaques ($P < .001$) (figure 6*B*).

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 immunoprophylaxis [14]. If another spore release

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 as 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 2*A*), 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 ED_{50}) 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-

hibited serum TNA antibody levels of ~5000 ED_{50} 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 5*A* and 6*A*), 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.

Acknowledgments

We thank James Panek and Marc Gurwith of VaxGen, for providing the developmental PA83 lots for immunization, and Christopher K. Allen and Susan Garges, for providing the BioThrax (Emergent Biosolutions) vaccine. We also thank Ivan Tatarov and Theresa M. Alexander (Veterinary Resources, University of Maryland, Baltimore), for assistance in immunization experiments, and Aldo Resendiz-Albor, Seema Thayil, and Mardi Reymann (Applied Immunology Section, Center for Vaccine Development, University of Maryland), for outstanding technical support.

References

1. Jernigan DB, Raghunathan PL, Bell BP, et al. Investigation of bioterrorism-related anthrax, United States, 2001: epidemiologic findings. Emerg Infect Dis **2002**; 8:1019–28.
2. Cieslak TJ, Kortepeter MG, Eitzen EM Jr. Vaccines against agents of bioterrorism. In: Levine MM, Kaper JB, Rappuoli R, Liu MA, Good MF, eds. New generation vaccines. 3rd ed. New York: Marcel Dekker, **2004**: 1067–79.
3. Hanson JF, Taft SC, Weiss AA. Neutralizing antibodies and persistence of immunity following anthrax vaccination. Clin Vaccine Immunol **2006**; 13:208–13.
4. Vitale L, Blanset D, Lowy I, et al. Prophylaxis and therapy of inhalational anthrax by a novel monoclonal antibody to protective antigen that mimics vaccine-induced immunity. Infect Immun **2006**; 74:5840–7.
5. Phipps AJ, Premanandan C, Barnewall RE, Lairmore MD. Rabbit and nonhuman primate models of toxin-targeting human anthrax vaccines. Microbiol Mol Biol Rev **2004**; 68:617–29.
6. Little SF, Ivins BE, Fellows PF, Pitt ML, Norris SL, Andrews GP. Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. Vaccine **2004**; 22:422–30.
7. Pitt ML, Little S, Ivins BE, et al. In vitro correlate of immunity in an animal model of inhalational anthrax. J Appl Microbiol **1999**; 87:304.
8. Williamson ED, Hodgson I, Walker NJ, et al. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. Infect Immun **2005**; 73:5978–87.
9. Brey RN. Molecular basis for improved anthrax vaccines. Adv Drug Deliv Rev **2005**; 57:1266–92.
10. Grabenstein JD. Countering anthrax: vaccines and immunoglobulins. Clin Infect Dis **2008**; 46:129–36.
11. Joellenbeck LM, Zwanziger LL, Durch JS, Strom BL. The anthrax vaccine: is it safe? Does it work? Washington, DC: National Academy Press, **2002**.
12. Payne DC, Rose CE Jr, Aranas A, et al. Assessment of anthrax vaccination data in the Defense Medical Surveillance System, 1998–2004. Pharmacoepidemiol Drug Saf **2007**; 16:605–11.
13. Martin SW, Tierney BC, Aranas A, et al. An overview of adverse events reported by participants in CDC's anthrax vaccine and antimicrobial availability program. Pharmacoepidemiol Drug Saf **2005**; 14:393–401.
14. Fowler GL, Baggs JM, Weintraub ES, Martin SW, McNeil MM, Gust DA. Factors influencing laboratory workers' decisions to accept or decline anthrax vaccine adsorbed (AVA): results of a decision-making study in CDC's anthrax vaccination program. Pharmacoepidemiol Drug Saf **2006**; 15:880–8.
15. Nass M. The Anthrax Vaccine Program: an analysis of the CDC's recommendations for vaccine use. Am J Public Health **2002**; 92:715–21.
16. US General Accounting Office. Anthrax vaccine. GAO's survey of guard and reserve pilots and aircrew. Report GAO-02–445. Washington, DC: US General Accounting Office, **2002**.
17. Little SF, Ivins BE, Webster WM, Norris SL, Andrews GP. Effect of aluminum hydroxide adjuvant and formaldehyde in the formulation of rPA anthrax vaccine. Vaccine **2007**; 25:2771–7.
18. Campbell JD, Clement KH, Wasserman SS, Donegan S, Chrisley L, Kotloff KL. Safety, reactogenicity and immunogenicity of a recombinant protective antigen anthrax vaccine given to healthy adults. Hum Vaccin **2007**; 3:205–11.
19. Gorse GJ, Keitel W, Keyserling H, et al. Immunogenicity and tolerance of ascending doses of a recombinant protective antigen (rPA102) anthrax vaccine: a randomized, double-blinded, controlled, multicenter trial. Vaccine **2006**; 24:5950–9.
20. Sambrook J, MacCallum P, Russell D. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, **2001**.
21. Tacket CO, Sztein MB, Wasserman SS, et al. Phase 2 clinical trial of attenuated *Salmonella enterica* serovar Typhi oral live vector vaccine CVD 908-*htrA* in U.S. volunteers. Infect Immun **2000**; 68:1196–201.
22. Galen JE, Chinchilla M, Zhao L, et al. Adaptation of the endogenous *Salmonella enterica* serovar Typhi *clyA*-encoded hemolysin for antigen export enhances the immunogenicity of anthrax protective antigen domain 4 expressed by the attenuated live vector vaccine strain CVD 908-*htrA*. Infect Immun **2004**; 72:7096–106.

23. Quinn CP, Dull PM, Semenova V, et al. Immune responses to *Bacillus anthracis* protective antigen in patients with bioterrorism-related cutaneous or inhalation anthrax. J Infect Dis **2004**; 190:1228–36.
24. Li H, Soroka SD, Taylor TH Jr, et al. Standardized, mathematical model-based and validated in vitro analysis of anthrax lethal toxin neutralization. J Immunol Methods **2008**; 333:89–106.
25. Crockett M, Keystone J. "I hate needles" and other factors impacting on travel vaccine uptake. J Travel Med **2005**; 12(Suppl 1):S41–6.
26. Nir Y, Paz A, Sabo E, Potasman I. Fear of injections in young adults: prevalence and associations. Am J Trop Med Hyg **2003**; 68:341–4.
27. Tacket CO, Galen J, Sztein MB, et al. Safety and immune responses to attenuated *Salmonella enterica* serovar Typhi oral live vector vaccines expressing tetanus toxin fragment C. Clin Immunol **2000**; 97:146–53.
28. Levine MM, Taylor DN, Ferreccio C. Typhoid vaccines come of age. Pediatr Infect Dis J **1989**; 8:374–81.
29. Kang HY, Curtiss R III. Immune responses dependent on antigen location in recombinant attenuated *Salmonella typhimurium* vaccines following oral immunization. FEMS Immunol Med Microbiol **2003**; 37: 99–104.
30. Hess J, Gentschev I, Miko D, et al. Superior efficacy of secreted over somatic antigen display in recombinant *Salmonella* vaccine induced protection against listeriosis. Proc Natl Acad Sci USA **1996**; 93:1458–63.
31. Galen JE, Levine MM. Can a 'flawless' live vector vaccine strain be engineered? Trends Microbiol **2001**; 9:372–6.
32. Stokes MG, Titball RW, Neeson BN, et al. Oral administration of a *Salmonella enterica*-based vaccine expressing *Bacillus anthracis* protective antigen confers protection against aerosolized *B. anthracis.* Infect Immun **2007**; 75:1827–34.
33. Pittman PR, Norris SL, Barrera Oro JG, Bedwell D, Cannon TL, McKee KT Jr. Patterns of antibody response in humans to the anthrax vaccine adsorbed (AVA) primary (six-dose) series. Vaccine **2006**; 24:3654–60.
34. Calvas P, Apoil P, Fortenfant F, et al. Characterization of the three immunoglobulin G subclasses of macaques. Scand J Immunol **1999**; 49: 595–610.
35. Semenova VA, Schmidt DS, Taylor TH Jr, et al. Analysis of anti-protective antigen IgG subclass distribution in recipients of anthrax vaccine adsorbed (AVA) and patients with cutaneous and inhalation anthrax. Vaccine **2007**; 25:1780–8.