A Three-Dose Intramuscular Injection Schedule of Anthrax Vaccine Adsorbed Generates Sustained Humoral and Cellular Immune Responses to Protective Antigen and Provides Long-Term Protection against Inhalation Anthrax in Rhesus Macaques

Conrad P. Quinn,[a] Carol L. Sabourin,[b] Nancy A. Niemuth,[b] Han Li,[a] Vera A. Semenova,[a] Thomas L. Rudge,[b] Heather J. Mayfield,[b] Jarad Schiffer,[a] Robert S. Mittler,[c,d] Chris C. Ibegbu,[c,d] Jens Wrammert,[c] Rafi Ahmed,[c] April M. Brys,[b] Robert E. Hunt,[b] Denyse Levesque,[d] James E. Estep,[b] Roy E. Barnewall,[b] David M. Robinson,[b] Brian D. Plikaytis,[a] and Nina Marano[a] for the AVRP Laboratory Working Group

Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA[a]; Battelle Biomedical Research Center, Columbus, Ohio, USA[b]; Emory Vaccine Center, Emory University, Atlanta, Georgia, USA[c]; and Yerkes National Primate Research Center, Emory University, Atlanta, Georgia, USA[d]

A 3-dose (0, 1, and 6 months) intramuscular (3-IM) priming series of a human dose (HuAVA) and dilutions of up to 1:10 of anthrax vaccine adsorbed (AVA) provided statistically significant levels of protection (60 to 100%) against inhalation anthrax for up to 4 years in rhesus macaques. Serum anti-protective antigen (anti-PA) IgG and lethal toxin neutralization activity (TNA) were detectable following a single injection of HuAVA or 1:5 AVA or following two injections of diluted vaccine (1:10, 1:20, or 1:40 AVA). Anti-PA and TNA were highly correlated (overall $r^2 = 0.89$ for $\log_{10}$-transformed data). Peak responses were seen at 6.5 months. In general, with the exception of animals receiving 1:40 AVA, serum anti-PA and TNA responses remained significantly above control levels at 28.5 months (the last time point measured for 1:20 AVA), and through 50.5 months for the HuAVA and 1:5 and 1:10 AVA groups ($P < 0.05$). PA-specific gamma interferon (IFN-γ) and interleukin-4 (IL-4) CD4$^+$ cell frequencies and T cell stimulation indices were sustained through 50.5 months (the last time point measured). PA-specific memory B cell frequencies were highly variable but, in general, were detectable in peripheral blood mononuclear cells (PBMC) by 2 months, were significantly above control levels by 7 months, and remained detectable in the HuAVA and 1:5 and 1:20 AVA groups through 42 months (the last time point measured). HuAVA and diluted AVA elicited a combined Th1/Th2 response and robust immunological priming, with sustained production of high-avidity PA-specific functional antibody, long-term immune cell competence, and immunological memory (30 months for 1:20 AVA and 52 months for 1:10 AVA). Vaccinated animals surviving inhalation anthrax developed high-magnitude anamnestic anti-PA IgG and TNA responses.

Anthrax vaccine adsorbed (AVA; BioThrax) was licensed in the United States in 1970 for prevention of anthrax in humans (30, 37, 56, 58). AVA is prepared from sterile culture filtrates of the toxigenic, nonencapsulated *Bacillus anthracis* strain V770-NP1-R grown under microaerophilic conditions in a chemically defined protein-free medium. The final product is formulated to contain 1.2 mg/ml aluminum (as aluminum hydroxide) in 0.85% sodium chloride, with 25 μg/ml benzethonium chloride and 100 μg/ml formaldehyde added as preservatives (58). The primary immunogen in AVA is anthrax toxin protective antigen (PA). Anti-PA IgG antibodies are considered to protect against anthrax by neutralizing the toxin, inhibiting spore germination, and enhancing phagocytosis and killing of spores by macrophages (1, 11, 12, 16, 39, 42, 49, 50, 55, 56).

The AVA schedule was recently approved as three 0.5-ml intramuscular (i.m.) injections, at 0, 1, and 6 months, with subsequent boosters at 12 and 18 months and annually thereafter for those at continued risk of infection (http://www.fda.gov/BiologicsBlood Vaccines/Vaccines/ApprovedProducts/ucm304758.htm). These and other recent changes in the use of AVA subsequent to the 1970 schedule were based on data from the Centers for Disease Control and Prevention (CDC) Anthrax Vaccine Research Program (AVRP) (30). The AVRP comprised a phase 4 human clinical trial to assess the safety and serological noninferiority of reduced schedules and parenteral AVA injection. The low prevalence of

anthrax in humans, however, precluded field efficacy studies of AVA. Therefore, to evaluate the potential efficacy of reduced AVA schedules and to build comprehensive immunogenicity profiles for AVA, the CDC AVRP included a rhesus macaque nonhuman primate (NHP) study of vaccine effectiveness and immunological correlates of protection (COP).

The AVRP human clinical trial was initiated in 1999 and included as its minimum schedule an i.m. priming schedule of 3 doses, at months 0, 1, and 6 (3-IM) (30), with a 3-year (42 months) booster (4-IM) (J. G. Wright and coworkers, submitted for publication). The AVRP did not provide field efficacy data. Therefore, the objectives of the NHP study were to modulate the NHP immune response by using a range of AVA dilutions, to evaluate the levels of protection afforded by diluted AVA from the 3-IM minimal priming series at key time points in the human

TABLE 1 Rhesus macaque AVA vaccination, challenge schedule, and survival data[a]

Group	Vaccine dilution	Challenge time (mo)	Avg challenge LD$_{50}$	No. of survivors/total no. of animals challenged (%)[a]	Fisher's exact test comparison to saline controls (P value)
1	HuAVA (undiluted)	52	403	8/10 (80.0)	0.020
2	1:5	52	506	9/9 (100)	0.001
3	1:10	52	514	6/10 (60.0)	0.139
4	1:20	12	293	5/10 (50.0)	0.433
5	1:40	12	37	4/10 (40.0)	0.696
6	1:10	12	380	8/10 (80.0)	0.020
7	1:20	12	366	6/10 (60.0)	0.139
8	1:40	12	396	9/10 (90.0)	0.002
9	1:10	30	840	6/9 (66.7)	0.109
10	HuAVA (undiluted)	30	573	10/10 (100)	0.001
11	1:5	30	504	8/8 (100)	0.001
12	1:20	30	509	7/8 (87.5)	0.011
Saline controls[b]	Saline	Based on paired group		7/23 (30.4)	
Process controls[c]	Not vaccinated	30	672	2/12 (16.7)	
	Not vaccinated	52	672	2/10 (20.0)	

[a] Vaccinated animals received AVA (0.5 ml) on a 3-dose (0, 1, and 6 months) i.m. schedule with either a full human dose (HuAVA) or saline-diluted AVA (1:5, 1:10, 1:20, or 1:40). Delivered doses of aerosolized *B. anthracis* spores ranged from 37 to 840 LD$_{50}$ equivalents (median, 504; mean, 461; geometric mean, 391) at the 3 challenge time points. Blank cells in the table indicate that the data were not applicable.

[b] Two saline controls were assigned the same injection, blood draw, and challenge schedule as each study group. The controls were combined and considered a separate group for analysis. For reasons unrelated to treatment, one saline control NHP was euthanized before challenge.

[c] Process controls were added at the 30- and 52-month challenge time points to increase statistical power for comparison to vaccinated animal survival at fixed challenge times. Process control animals did not have prechallenge blood draws.

vaccination booster schedule, to assess the duration of protection provided by the 3-IM priming by infectious challenge of NHPs at 52 months, and to establish immunological response profiles to AVA that might facilitate the determination of immune correlates of protection for this vaccine. The 12-month and 30-month challenge time points in the NHP study corresponded to the human booster schedule, with vaccinations replaced by saline injection. The human and NHP vaccination and challenge schedules are compared in Table SA1 in the supplemental material.

We report the following for NHPs: the duration of protection provided by the 3-dose priming series (3-IM), a characterization of the vaccine-induced humoral anti-PA IgG and lethal toxin neutralization activity (TNA) responses, an overview of T and B cell immune responses, the Th1/Th2 disposition, an assessment of anti-PA IgG and TNA postexposure responses to aerosolized spores of *B. anthracis* Ames, and an analysis of the COP for AVA in rhesus macaques.

MATERIALS AND METHODS

Nonhuman primate study design, vaccination schedule, and challenge. To modulate the immune responses and provide a gradation of survival frequencies at the 12-, 30-, and 52-month challenge time points, rhesus macaques (*Macaca mulatta*) were vaccinated with a range of AVA dilutions in sterile saline. Dilutions in saline maintained the antigen/adjuvant ratio. Vaccinated animals (*n* = 17 to 30 per group) received AVA (0.5 ml) on a 3-dose (0, 1, and 6 months) i.m. schedule, using either the full human dose (HuAVA) or saline-diluted AVA (1:5, 1:10, 1:20, or 1:40). Due to the number of vaccinated NHPs (*n* = 114) and the long duration and multisite nature of the study, the NHPs were organized into 12 groups of 8 to 10 animals each. Control animals received saline injections (*n* = 23) or were untreated process controls (*n* = 22) (Table 1). In anticipation of immunity waning in NHPs receiving higher dilutions of AVA, the study incorporated a planned imbalance. Groups with higher vaccine dilutions were challenged earlier in the study, and groups with lower vaccine dilutions were challenged later, with crossover in the 1:5, 1:10, and 1:20 AVA groups (see Tables 2 and 4). To complete the vaccination of all animals, two lots of AVA were used: groups 1 to 5 received lot FAV048B, and groups 6 to 12 received lot FAV074. Groups 1 to 9 were vaccinated at the Battelle Biomedical Research Center (BBRC), Columbus, OH. Groups 10 to 12 were vaccinated at the Yerkes National Primate Research Center, Emory University, Atlanta, GA, and were relocated to the BBRC for infection studies. NHPs were exposed to a target dose of 200 or 400 50% lethal dose (LD$_{50}$) equivalents of aerosolized *B. anthracis* Ames spores 12, 30, or 52 months after the first vaccination (Table 1). Prechallenge blood and serum samples were collected from vaccinated and saline control animals (Table 2). Samples available for postexposure (PE) analysis were from PE days 3, 5, 7, 14, and 30 for 1:10, 1:20, and 1:40 AVA groups challenged at 12 months (groups 4 to 8) and from PE days 0, 14, and 30 for HuAVA and 1:5, 1:10, and 1:20 AVA groups challenged at 30 and 52 months (groups 1 to 3 and 9 to 12). Aerosol challenge and blood collection protocols have been described elsewhere (4).

All aspects of the study were designed to minimize stress to the animals. Animals judged to be moribund after spore challenge were humanely euthanized. All animal study procedures were compliant with all relevant federal guidelines and were approved by the Institutional Animal Care and Use Committees (IACUCs) of the BBRC, Emory University, and the CDC.

Protective antigen and standard reference sera. Recombinant protective antigen (rPA) and recombinant lethal factor (rLF) were obtained from BEI Resources (Manassas, VA). The murine monocyte/macrophage cell line J774A.1 (TIB-67) was obtained from the American Type Culture Collection (ATCC), Manassas, VA. The preparation and characterization of rhesus macaque reference serum AVR731, used for anti-PA enzyme-linked immunosorbent assay (ELISA), and human reference standard AVR801, used in the TNA assay, are described elsewhere (20, 44).

Rhesus macaque standard reference serum BMI269 for IgG subclass determination was prepared by pooling sera from animals that survived challenge with *B. anthracis* strain Vollum and were subsequently vaccinated with AVA.

TABLE 2 Scheduled prechallenge blood draws for rhesus macaques vaccinated with AVA[a]

Sample point	Time of blood draw (mo post-first vaccination)				
	Groups 1–3, challenged at 52 mo	Groups 4–5, challenged at 12 mo	Groups 6–8, challenged at 12 mo	Group 9, challenged at 30 mo	Groups 10–12, challenged at 30 mo
1	0	0	0	0	0
2	1	1	0.5	0.5	1
3	2	2	1	1	2
4	4*	NS	2	2	3
5	5*	NS	3	3	4
6	6	6	4	4	5
7	6 PP day 3*	6 PP day 3*	5	5	6
8	6 PP day 5*	6 PP day 5*	6	6	6 PP day 3*
9	6 PP day 7*	6 PP day 7*	6 PP day 3*	6 PP day 3	6 PP day 6*
10	6 PP day 14*	6 PP day 14*	6 PP day 5*	6 PP day 7	6 PP day 10*
11	7	7	6 PP day 7*	6 PP day 14	6 PP day 14*
12	8	8	6 PP day 14*	7	7
13	9	9	7	8	8
14	10	10	8	9	9
15	11	11	9	10	10
16	11.75	11.75	10	11	11
17	12 (group 3 only)	12 (group 5 only), NS (group 4)	11	11.75	11.75
18	12.5		11.75	12	12.5
19	13 (group 3 only)			15.5	15.5
20	15.5			18	18
21	18			21	21
22	21			24	24
23	24			27.25	26.25
24	27.25				28.5
25	30				
26	33				
27	36				
28	38.75				
29	41.75				
30	44.75				
31	47.5				
32	50.5				

[a] Data from groups that received the same AVA dilution dose were combined for immunological analysis when the blood draw schedules coincided. Blank cells indicate that samples were not scheduled for the indicated collection times. NS, no sample available; PP, post-priming vaccination at month 6 (serum only); *, time point where only serum samples were scheduled to be collected.

Anti-PA IgG ELISA. Immulon 2 HB microtiter plates (Thermo Labsystems, Franklin, MA) were coated with rPA (2 μg/ml) in phosphate-buffered saline (PBS), pH 7.4 (Life Technologies, Gaithersburg, MD). Plates were washed 3 times with PBS containing 0.1% Tween 20. Test sera were added to wells preloaded with 100 μl of PBS containing 5% (wt/vol) skim milk and 0.5% (vol/vol) Tween 20, pH 7.4, mixed in the plate, and serially transferred to make an 8-point dilution series with 100 μl/well. After washing, bound anti-PA IgG was detected with horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG (Research Diagnostics, Inc., Flanders, NJ), and color was developed with ABTS substrate [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); Kirkegaard and Perry Laboratories, Gaithersburg, MD]. Data were analyzed using a four-parameter logistic-log (4-PL) curve-fitting model with ELISA for Windows software (version 2.15). Reportable values of anti-PA IgG were obtained in μg/ml, using a calibration factor of 171.9 μg/ml for reference serum AVR731. The lower limits of detection (LOD) and quantification (LLOQ) were 0.4 and 2.3 μg/ml anti-PA IgG, respectively.

Anti-PA subclass ELISAs. Although the genes for 4 IgG subclasses analogous to human IgG subclasses 1 to 4 have been identified (43), rhesus macaques are reported to produce only 3 IgG subclasses, analogous to human IgG1, IgG2, and IgG4 (45). Detection and quantification of each of the NHP anti-PA IgG subclasses in this study were done in separate assays using anti-human monoclonal antibodies specific for each of the 4 human IgG subclasses (mouse anti-human IgG1 [05-3300; Zymed Laboratories Inc., South San Francisco, CA]; mouse anti-human IgG2 [ab1933; Novus Biologicals, Littleton, CO], mouse anti-human IgG3 [ab1928; Novus Biologicals], and mouse anti-human IgG4 [YNMAHIgG1-4SET; Accurate Chemical Company, Westbury, NY]). A conjugate HRP-labeled sheep anti-mouse gamma chain IgG (515-035-062; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used for IgG1, and HRP-labeled donkey anti-mouse gamma chain IgG (715-035-150; Jackson Immuno-Research Laboratories, Inc.) was used for IgG subclasses 2, 3, and 4. Assays for all 4 IgG subclasses were run when the total anti-PA IgG titer was ≥5 μg/ml. The anti-PA IgG subclass concentrations were calculated in ng/ml by interpolation to the calibration curve, using a 4-PL model and SoftMax Pro software (version 4.3). Reportable values for anti-PA IgG1, IgG2, IgG3, and IgG4 were obtained in μg/ml, using calibration factors of 258, 57.8, 9.00, and 4.88 μg/ml, respectively, for reference serum BMI269, calibrated from AVR801 (44). The LODs for IgG subclasses were 20.2, 18.1, 5.6, and 1.6 ng/ml for IgG1, IgG2, IgG3, and IgG4, respectively.

Relative subclass proportions in a sample were determined when the total anti-PA IgG titer was ≥ 10 µg/ml, all four subclasses were evaluated, and at least one IgG subclass was detected.

Anti-PA IgG avidity. Serum samples with total anti-PA IgG titers of ≥ 5 µg/ml were evaluated for avidity, an indirect assessment of polyclonal antibody affinity and immune response maturation and a surrogate for memory B cell persistence (28). The avidity indices (AI) were determined by anti-PA IgG elution from immobilized rPA with ammonium thiocyanate (NH_4SCN; 0.078 to 5 M) (Sigma). A 4-PL dissociation curve was generated for the percent maximum detected signal versus NH_4SCN concentration, and the AI is reported as the concentration (molar) of NH_4SCN required to elute 50% of bound anti-PA IgG.

TNA assay. TNA assays were done according to the method of Li et al. (20), using human reference standard AVR801 (44). Reportable values were the reciprocal serum sample dilutions effecting 50% neutralization of anthrax lethal toxin (ED_{50}). Endpoints were calculated using SAS, version 9.0 (SAS Institute Inc., Cary, NC). The LOD and LLOQ were ED_{50}s of 12 and 36, respectively (20). ED_{50} values of >0 were included in the statistical analyses.

Detection of IFN-γ- and IL-4-secreting cells. Peripheral blood mononuclear cells (PBMC) were prepared as described previously (32). Gamma interferon (IFN-γ)- and interleukin-4 (IL-4)-producing cells were enumerated by enzyme-linked immunosorbent spot (ELISpot) assay following *in vitro* restimulation with 1 µg/ml rPA (24 h for IFN-γ assays and 36 h for IL-4 assays). Staphylococcal enterotoxin B at 2 µg/well (Toxin Technology, Sarasota, FL) was used as a positive control. Unstimulated cultures served as negative controls. The frequency of IFN-γ$^+$ or IL-4$^+$ T cells specific for rPA was calculated by subtracting the average number of spot-forming units (SFU) in unstimulated negative-control triplicate wells from the average number of SFU in rPA-stimulated triplicate wells and expressed as the number of rPA-specific IFN-γ or IL-4 SFU/10^6 PBMC.

Lymphocyte stimulation indices. PBMC were plated in quadruplicate into 96-well round-bottom microtiter plates containing 200 µl of either medium alone or medium containing 1.25 µg/ml rPA. The positive control was phytohemagglutinin (10 µg/ml). Cells were incubated for 96 h at 37°C and 5% CO_2. Cultures were then pulsed with 20 µl of a 50-µCi/ml [^{3}H]thymidine solution and incubated for 18 h at 37°C and 5% CO_2. Cells were harvested onto filter discs (Fisher, Pittsburgh, PA) and counted on a Packard scintillation counter (Packard, Meriden, CT). Stimulation indices (SI) were calculated as the following quotient: mean counts per minute of stimulated cells/mean counts per minute of unstimulated cells.

Anti-PA IgG-specific B cells. Antigen-specific B cells were enumerated by ELISpot assay as described in detail elsewhere (7, 8) and modified for the proliferation and detection of rhesus macaque IgG-secreting cells. Macaque PBMC were plated in 24-well plates at 5×10^5 cells/well in R-10 medium supplemented with a mix of polyclonal mitogens: 1/10,000 pokeweed mitogen extract, 6 µg/ml CpG ODN-2006, and 1/10,000 *Staphylococcus aureus* strain Cowan (SAC) (Sigma). Cells were cultured for 6 days at 37°C and 6 to 8% CO_2. For ELISpot detection, 96-well filter plates (Millipore) were coated overnight with rPA at 1 µg/ml. Keyhole limpet hemocyanin (KLH) (2.5 µg/ml) was used as an antigen control. Total and rPA-specific IgG-secreting cells were detected using 10 µg/ml goat anti-monkey immunoglobulin (Accurate Chemical Co.). Data are presented as the frequency (percentage) of rPA-specific anti-PA-secreting cells versus total IgG-secreting cells in PBMC. The LOD was 0.002 antigen-specific IgG-secreting cell per 10^6 PBMC.

Statistical analyses. Humoral antibody (total anti-PA IgG, anti-PA IgG subclasses, avidity indices, and TNA ED_{50}s) and cellular (T cell proliferation and frequencies of memory B cells and IFN-γ- and IL-4-secreting cells) responses were analyzed using analysis of variance (ANOVA) models to compare the average response within each AVA vaccine dose group to that of the saline control group and to evaluate dose-response trends. Logistic regression models were used to determine whether the

humoral and cellular responses were predictive of survival of inhalation anthrax. All ANOVA and logistic regression models were fit separately to each parameter at each time point. All models were fit using the SAS, version 9.1.3, GLM, MIXED, PROBIT, and LOGISTIC procedures. All statistical tests were performed at the 0.05 level of significance, with adjustments for multiple comparisons.

Data from groups that received the same AVA dilution dose were combined for immunological analyses when the blood draw schedules coincided. To accommodate minor scheduling differences, weeks 34 and 35 (8 months), 46 and 47 (11 months), 50 and 51 (11.75 months), 65 through 67 (15.5 months), 78 through 80 (18 months), 90 through 92 (21 months), 102 through 105 (24 months), and 117 and 118 (27.25 months) were binned for analysis. Control animal data included in the statistical analysis were from the saline-injected controls; no prechallenge blood draws were collected for process controls. Analysis of PE immune responses included blood draws collected at PE days 14 and 30 for process control animals. Missing values were generated when there was insufficient sample for analysis or when samples were lost due to experimental failure. All valid results were included in the analysis. No attempt was made to impute missing values. Reportable values between the LOD and LLOQ were included in the statistical analysis. Values at or below the LOD were assigned a value equal to one-half the LOD (14). For statistical analysis of the TNA and avidity assays, all values of >0 were included, and zero values were replaced with an arbitrary nominal value that was less than the smallest measured value. For total anti-PA IgG assay analyses, the LOD was 0.4 µg/ml.

Avidity and IgG subclass assays were run if the total anti-PA IgG titer was ≥ 5 µg/ml. IgG subclass values were set to one-half the LOD when the total anti-PA IgG was detectable but was <5 µg/ml. If an IgG subclass assay was performed and the IgG subclass level was lower than the LOD, the reportable value was also set to one-half the LOD. If there was insufficient sample to run some or all of the subclass assays, then the subclass IgG values that were not measured were treated as missing values and not included in the analysis.

Evaluation of effects of vaccine dose and challenge time on survival. Prior to building logistic regression models for analysis of immunological COP, it was important to determine whether the vaccine dose and challenge time had significant effects on survival and subsequently to decide their inclusion or exclusion from the models. Fisher's exact test was used to determine significant differences in survival frequencies between AVA-vaccinated and saline control animals for individual study groups and for combined AVA dilution groups at each of the different challenge times. Fisher's exact test was also used to determine significant differences in survival frequencies (i) between vaccinated NHP groups that received the same AVA dilution but were challenged at different times, (ii) between vaccinated NHP groups that received the same AVA dilution and were challenged at the same nominal time interval but at different points in the study, and (iii) between challenge times with all vaccinated animals considered a single group. Logistic regression models of survival were fit in order to assess the relationship of challenge time and infectious dose to survival.

Logistic regression models of survival and immune response. Based on Fisher's exact test comparisons and the logistic regression models of vaccine dose and challenge time with survival, the data were combined across challenge times for logistic regression models used to relate total anti-PA IgG, anti-PA IgG subclasses, TNA, and SI to survival. The following logistic regression model was fit to each parameter by month to determine if the parameter was predictive of survival: $\ln[p/(1 - p)] = \beta_0 + \beta_1 \log_{10}(Y_i) + \varepsilon_i$, where p is the probability of survival, $\log_{10}(Y_i)$ is the observed $\log_{10}$-transformed parameter result for the ith animal, β_1 and β_0 are the slope and intercept of the logistic regression equation, respectively, and ε_i is the random error unexplained by the model. The model was fit by both including and excluding control animals for a subset of parameters, with similar results. Control animals were excluded from the final analysis. A statistically significant ($P < 0.05$) slope indicated a significant effect

of the log-adjusted parameter response on survival. The intercept represents the log odds of animals with a parameter value of 1 surviving the challenge. The models were used to estimate the parameter values associated with an 80% probability of survival as a metric for an acceptable level of vaccine effectiveness (48). Values greater than 10 times the maximum value observed at the modeled study time were not reported. The Hosmer-Lemeshow (H-L) test was used to evaluate goodness of fit for the logistic regression models. Receiver operating characteristic (ROC) analysis was used to estimate the predictive value of the model. The area under the ROC curve is the probability that the logistic model correctly predicted any randomly chosen survivor to be more likely to survive than any randomly chosen animal that died.

Correlation of anti-PA IgG and TNA. Linear models were used to establish the correlation between $\log_{10}$-transformed anti-PA IgG (μg/ml) and the TNA (ED_{50}) and to determine whether the correlation varied with vaccine dilution, number of doses, and time. The model included vaccine dilution-specific intercepts, an overall slope relating anti-PA IgG to ED_{50}, and dilution-specific adjustments to the overall slope. Analysis included all time points from 2 months onwards and all samples for which both the TNA ED_{50} and total anti-PA IgG were higher than the LOD.

The modeling was performed in two stages. Initially, the linear model $\log_{10}(Y_{ijk}) = \beta_0 + \alpha_i + \gamma_j + \beta_1\log_{10}(x_{ijk}) + \tau_i\log_{10}(x_{ijk}) + \upsilon_j\log_{10}(x_{ijk}) + \eta_{ij}\log_{10}(x_{ijk}) + \zeta_k + \varepsilon_{ijk}$, was fit, where Y_{ijk} is the TNA ED_{50} for the kth subject that received the ith treatment (vaccine dose) at the jth time, β_0 is the intercept of the regression line, α_i is the effect of the ith treatment group on the intercept, γ_j is the effect of the jth time on the intercept, x_{ijk} is the observed total anti-PA IgG measured by ELISA for the kth subject that received the ith treatment at the jth time, β_1 is the slope of the regression line relating $\log_{10}$ total anti-PA IgG measured by ELISA to $\log_{10}$ TNA ED_{50}, and τ_i, υ_j, and η_{ij} are adjustments to the slope based on treatment group and time. The model also included a random subject effect, ζ_k, to account for repeated measurements on the same subjects, and an error term, ε_{ijk}, to account for random variation not explained by the model.

Evaluation of the initial model indicated that the effects of vaccine dilution dose and time were significant. For ease of interpretation, the final models were fit separately at each time point. The final model was $\log_{10}(Y_{ijk}) = \beta_0 + \alpha_i + \beta_1\log_{10}(x_{ijk}) + \tau_i\log_{10}(x_{ijk}) + \varepsilon_{ijk}$. The terms in this model are defined as described above. Because the models were fit separately at each time point, the random subject effect was not required. Least-squares means from the model were used for pairwise comparisons of $\log_{10}$ TNA ED_{50}s at the average value for $\log_{10}$ anti-PA IgG between groups at each study time point.

RESULTS

Anti-PA IgG responses. Significant linear vaccine dilution dose trends were seen from 1 month to 50.5 months (Fig. 1A). Anti-PA IgG levels 4 weeks after each vaccination (1, 2, and 7 months) and the peak response at 6.5 months are shown in Table 3. The HuAVA (64.3 μg/ml; 95% confidence interval [95% CI], 37.6 to 109.8μg/ml; 20/20 samples were above the LOD) and 1:5 AVA (1.8 μg/ml; 95% CI, 0.98 to 3.4 μg/ml; 18/20 samples were above the LOD) groups had detectable anti-PA IgG by 1 month (Table 3). The second AVA immunization, at 1 month, resulted in increases in anti-PA IgG at 2 months for all AVA dilutions (range, 1.8 to 420.3 μg/ml) that were significantly different from controls ($P < 0.05$). The anti-PA IgG geometric mean concentration (GMC) decreased but remained detectable to 6 months in all groups except the 1:40 AVA group (for the 1:20 AVA group, 0.52 μg/ml, 95% CI of below detection to 0.75 μg/ml, and 18/30 samples were above the LOD; for the 1:10 AVA group, 1.6 μg/ml, 95% CI of 1.1 to 2.2 μg/ml, and 28/30 samples were above the LOD; for the 1:5 AVA group, 5.5 μg/ml, 95% CI of 3.3 to 9.3 μg/ml, and 20/20 samples were above the LOD; and for the HuAVA group,
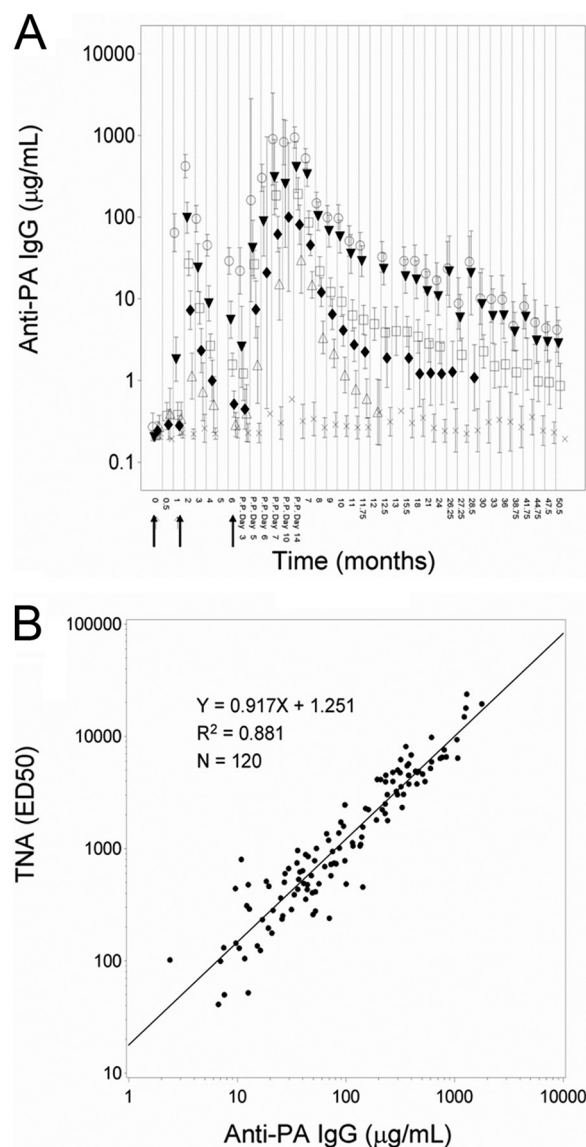

FIG 1 Humoral antibody responses to a 3-IM schedule of AVA. Vaccinated rhesus macaques received AVA (0.5 ml) on a 3-dose (0, 1, and 6 months) i.m. schedule with either HuAVA or a 1:5, 1:10, 1:20, or 1:40 AVA dilution. Vertical arrows indicate injection time points. PP, days after priming at 6 months. Control animals received saline injections. (A) Anti-PA IgG GMC (μg/ml) and 95% CI for each dose group on each study day. (B) Linear regression analysis of TNA (ED_{50}) versus anti-PA IgG (μg/ml) postpriming at 7 months ($n = 120$ observations). The equation for the best fit line is shown ($r^2 = 0.881$). ○, HuAVA; ▼, 1:5 AVA; □, 1:10 AVA; ◆, 1:20 AVA; △, 1:40 AVA; ×, controls.

29.1 μg/ml, 95% CI of 20.0 to 42.2 μg/ml, and 20/20 samples were above the LOD).

A subset of study NHPs was analyzed at days 3, 5, 6, 7, 10, and 14 after completing the priming series at 6 months. Groups 1 to 5 had blood draws at postpriming (PP) days 3, 5, 7, and 14; group 9 had blood draws at PP days 3, 7, and 14; and groups 10 to 12 had blood draws at PP days 3, 6, 10, and 14. By PP day 5, anti-PA IgG GMCs in all vaccine groups were significantly higher than that of controls. Anti-PA IgG GMCs continued to increase, with the highest levels observed at PP day 14 (6.5 months) (Table 3). After PP day 14, anti-PA IgG levels decreased with time, in a vaccine

TABLE 3 Rhesus macaque anti-PA IgG and TNA responses at 1, 2, 6.5, and 7 months of 3-IM priming with dilutions of AVA[a]

| Time point (mo) | Geometric mean value (95% CI) (n) | | | | | |
| | HuAVA | | 1:5 AVA | | 1:10 AVA | |
	Anti-PA IgG (μg/ml)	TNA ED_{50}	Anti-PA IgG (μg/ml)	TNA ED_{50}	Anti-PA IgG (μg/ml)	TNA ED_{50}
1	64.3 (37.6, 109.8) (20)	422 (251, 711) (20)	2.2 (1.3, 3.7) (20)	37 (25, 55) (20)	1.2 (1.1, 1.3) (30)	18 (*) (30)
2	420.3 (302.3, 584.4) (20)	3,611 (2,494, 5,227) (20)	98.1 (63.5, 151.5) (20)	1,186 (685, 2,052) (20)	27.1 (19.2, 38.2) (30)	306 (222, 421) (30)
6.5	943.1 (700.0, 1,270.7) (7)	11,306 (6,340, 20,162) (7)	409.3 (200.6, 835.0) (7)	4,950 (2,829, 8,659) (7)	192.5 (122.7, 302.1) (12)	2,184 (1,146, 4,166) (12)
7	523.3 (397.5, 689.0) (20)	5,283 (3,938, 7,089) (20)	333.6 (237.7, 468.0) (20)	3,790 (2,447, 5,870) (20)	85.8 (61.9, 119.2) (30)	1,091 (749, 1,590) (30)

[a] Time points of 1, 2, and 7 months were selected to illustrate responses to the preceding vaccinations at 0, 1, and 6 months, respectively, and for their correspondence to the CDC AVRP human clinical trial. The data for the 6.5-month time point represent the maximum responses measured at completion of the 3-IM priming. *, all values were at or below the LLOQ, and therefore no confidence interval could be calculated. n = number of animals tested at the indicated time point.

dilution dose-dependent manner. At 7 months, the GMCs ranged from 14.8 to 523.3 μg/ml, from the 1:40 AVA to HuAVA groups, respectively (Table 3).

At the last available measurement prior to the aerosol challenge time points at 12, 30, and 52 months, anti-PA IgG GMCs were significantly ($P < 0.05$) higher than the control group levels, with the exception of the 1:40 AVA group 5 at 12 months, where 4/10 animals had anti-PA IgG levels above the LOD. Anti-PA IgG GMCs at 50.5 months were as follows: for the 1:10 AVA group, 0.86 μg/ml, with 95% CI of 0.51 to 1.8 μg/ml and 9/10 samples above the LOD; for the 1:5 AVA group, 2.9 μg/ml, with 95% CI of 1.5 to 5.5 μg/ml and 9/9 samples above the LOD; and for the HuAVA group, 4.2 μg/ml, with 95% CI of 2.2 to 7.8 μg/ml and 10/10 samples above the LOD.

TNA assay results. Similar to total anti-PA IgG, serum TNA demonstrated significant linear vaccine dose dilution response trends for 1 through 50.5 months (not shown) that were highly correlated with anti-PA IgG levels (Fig. 1B). TNA levels 4 weeks after each vaccination (1, 2, and 7 months) and the peak response at 6.5 months are shown in Table 3. A single vaccination with HuAVA or 1:5 AVA elicited TNA at 1 month (for HuAVA group, geometric mean titer-ED_{50} [GMT-ED_{50}] = 423, 95% CI = 251 to 710, and 20/20 animals had a titer of >0; and for 1:5 AVA group, GMT-ED_{50} = 16.3, 95% CI = 6.5 to 41.2, and 14/20 animals had a titer of >0). The second AVA vaccination (1 month) resulted in a significant (>3-fold) increase in ED_{50} for all vaccination groups at 2 months (ED_{50} range, 3.6 to 3,610 for the 1:40 AVA to HuAVA groups, respectively). By 3 months, the GMT-ED_{50} in the 1:40 AVA vaccine group decreased to control levels but remained elevated in all other groups. Completion of the priming series at 6 months stimulated a rapid rise in GMT-ED_{50} at PP days 5, 7, and 14. At 7 months, GMT-ED_{50} values ranged from 211 to 5,283, from the 1:40 AVA to HuAVA groups, respectively (Table 3).

All AVA vaccine groups had ED_{50} titers significantly higher than those of controls for the study duration, with the exception of the 1:40 AVA group at 11.75 months (GMT-ED_{50} = 1.73, 95% CI = 0.91 to 3.3, and 3/20 animals had a titer of >0) and the 1:10 AVA group at 50.5 months (GMT-ED_{50} = 2.03, 95% CI = 0.69 to 6.0, and 2/10 animals had a titer of >0). Prior to the aerosol challenge time points, at 28.5 months the lowest TNA level significantly higher than that of controls was in the 1:20 AVA group (GMT-ED_{50} = 10.40, 95% CI = 2.6 to 41.3, and 6/9 animals had a titer of >0); at 30 months, the lowest significant TNA level was in the 1:10 AVA group (GMT-ED_{50} = 15.0, 95% CI = 5.7 to 39.1, and 13/19 animals had a titer of >0); and at 50.5 months, the lowest significant TNA levels were in the HuAVA and 1:5 AVA groups (for the HuAVA group, GMT-ED_{50} = 99.50, 95% CI =

53.1 to 187, and 10/10 animals had a titer of >0; and for the 1:5 AVA group, GMT-ED_{50} = 39.2, 95% CI = 12.6 to 122, and 8/9 animals had a titer of >0).

Correlation of anti-PA IgG with TNA. TNA and concentrations of anti-PA IgG were highly correlated during active immunization and at completion of immune priming (r^2 = 0.92 and 0.88 at 2 months and 7 months, respectively) (Fig. 1B, data for 7 months are shown). The slopes of linear regression models relating anti-PA IgG to ED_{50} were statistically significant at every sample collection time, and the model r^2 value was high (>0.87) throughout the first 24 months of the study. These data indicate a sustained anti-PA IgG *in vitro* functional activity.

In general, the relationship between quantity and *in vitro* neutralizing activity of anti-PA antibodies did not vary with vaccine dilution. With the exceptions of 2 months, 3 months, and 5 months, there were no significant differences among the dilution groups in the slope or intercept during the priming series. Where significant differences were detected, the slopes and intercepts appeared to interact such that the differences in predicted values between groups were unlikely to be biologically significant. In these instances during the priming series, the regression slopes were flatter for higher vaccine dilutions (1:20 and 1:40 AVA). In contrast, where significant differences were detected PP (12.5, 24, and 26.25 months), the slope for the 1:5 AVA dilution group was significantly steeper than that for the HuAVA group.

Anti-PA IgG subclasses. Anti-PA total IgG concentrations were sufficiently high for subclass analysis over a 36-month study period. All four subclasses were detectable using anti-human reagents. In general, anti-PA IgG subclass levels paralleled the total anti-PA IgG levels. The HuAVA and 1:5 AVA groups provided the most representative information for relative subclass proportions (Fig. 2A and data not shown). Anti-PA IgG1 and IgG2 were the most abundant subclasses, with IgG1 being predominant. The relative levels of IgG1 and IgG2 were similar at 6-month PP days 7 and 14 (6.5 months; peak response). However, only 7/22 samples obtained from the HuAVA and 1:5 AVA groups had detectable levels of all 4 subclasses at these time points.

Anti-PA IgG avidity. The first vaccination with HuAVA or 1:5 diluted AVA stimulated anti-PA IgG antibodies with a detectable AI at 1 month (for the HuAVA group, AI = 0.27 and 15/15 animals had an AI of >0; for the 1:5 AVA group, AI = 0.31 and 3/3 animals had an AI of >0) (Fig. 2B). The second dose (1 month) resulted in antibodies with increased mean avidity by 2 months. With the exception of the 1:40 AVA group, the AI values were similar for all vaccine dilution groups (range, 0.24 to 0.31). In the 1:40 AVA group at 2 months, the AI was 0.05 for the 2 samples that had sufficient total anti-PA IgG for assay. Anti-PA AI increased

TABLE 3 (Continued)

1:20 AVA		1:40 AVA		Controls	
Anti-PA IgG (μg/ml)	TNA ED$_{50}$	Anti-PA IgG (μg/ml)	TNA ED$_{50}$	Anti-PA IgG (μg/ml)	TNA ED$_{50}$
1.2 (1.1, 1.3) (30)	18 (*) (30)	1.3 (1.1, 1.5) (20)	18 (*) (20)	1.2 (*) (24)	18 (*) (24)
8.0 (5.0, 12.7) (30)	94 (58, 151) (30)	1.8 (1.2, 2.8) (20)	22 (17, 27) (20)	1.8 (*) (22)	18 (*) (22)
80.8 (46.5, 140.6) (12)	1,055 (589, 1,890) (12)	29.9 (11.7, 76.1) (8)	452 (143, 1,433) (8)	1.15 (*) (10)	18 (*) (10)
45.5 (33.7, 61.3) (30)	576 (419, 793) (30)	14.8 (10.2, 21.5) (20)	211 (145, 307) (20)	1.3 (1.0, 1.7) (24)	18 (*) (24)

approximately 2-fold in all groups for up to 6 months prior to the last vaccination (for the HuAVA group, AI = 0.58 and 19/19 animals had an AI of >0; for the 1:5 AVA group, AI = 0.48 and 12/12 animals had an AI of >0; and for the 1:10 AVA group, AI = 0.53 and 2/2 animals had an AI of >0), indicating maturation of the antibody response following 2 vaccinations. Up to PP day 14 following the third (6 months) AVA vaccination, there was a transient reduction in AI in the subset of NHPs tested. By 7 months, however, the AI returned to levels similar to those at month 6 for the HuAVA group (AI = 0.61). At 7 months, there were modest increases compared to 6 months for the 1:5 AVA (AI = 0.64; 20/20 animals had an AI of >0), 1:10 AVA (AI = 0.60; 29/29 animals had an AI of >0), 1:20 AVA (AI = 0.46; 30/30 animals had an AI of >0), and 1:40 AVA (AI = 0.49; 19/19 animals had an AI of >0) groups. High-avidity antibodies persisted in samples that were assayed through 33 months (AI range, 0.45 to 0.83) (Fig. 2B).

Lymphocyte stimulation indices. In contrast to anti-PA IgG and TNA, all vaccinated NHPs demonstrated a statistically significant increase in SI at 1 month (for HuAVA, mean SI of 16.0, 95% CI of 9.8 to 26.4, and 20/20 animals with SI of >0; for 1:5 AVA, mean SI of 8.3, 95% CI of 4.5 to 15.1, and 20/20 animals with SI of >0; for 1:10 AVA, mean SI of 3.9, 95% CI of 2.5 to 6.2, and 29/29 animals with SI of >0; for 1:20 AVA, mean SI of 3.2, 95% CI of 2.3 to 4.6, and 30/30 animals with SI of >0; and for 1:40 AVA, mean SI of 1.9, 95% CI of 1.4 to 2.4, and 18/18 animals with SI of >0), with a significant (>3-fold) increase in SI by 2 months (for HuAVA, mean SI of 53.5, 95% CI of 31.4 to 91.0, and 20/20 animals with SI of >0; for 1:5 AVA, mean SI of 42.6, 95% CI of 29.8 to 61.0, and 20/20 animals with SI of >0; for 1:10 AVA, mean SI of 18.6, 95% CI of 11.7 to 29.4, and 30/30 animals with SI of >0; for 1:20 AVA, mean SI of 12.4, 95% CI of 7.9 to 19.4, and 30/30 animals with SI of >0; and for 1:40 AVA, mean SI of 12.2, 95% CI of 6.5 to 22.8, and 20/20 animals with SI of >0) (Fig. 3A). Postpriming, the SI remained statistically higher than that for controls for all groups at all subsequent time points up to 50.5 months, with two exceptions: the 1:20 AVA group at 15.5 months and the HuAVA group at 30 months (time points for which background SI in the control groups were high).

Significant linear vaccine dose-related SI trends were seen from months 1 to 11.75, 12.5, 18, 20, 26.25, 28.5, and 33. For example, at 7 months, mean SI (95% CI) were as follows: for HuAVA, 71.5 (39.5 to 129.5), with 20/20 animals with SI of >0; for 1:5 AVA, 43.8 (23.9 to 80.3), with 20/20 animals with SI of >0; for 1:10 AVA, 28.8 (17.2 to 48.0), with 30/30 animals with SI of >0; for 1:20 AVA, 17.9 (10.4 to 30.9), with 30/30 animals with SI of >0; and for 1:40 AVA, 21.8 (11.3 to 41.8), with 19/19 animals with SI of >0. At the later time points, the SIs for the HuAVA and 1:5 AVA groups were similar (30.7 and 31.6, respectively, at 50.5 months). These data indicate that PA-specific effector lymphocyte compe-

tence was initiated following a single i.m. dose of AVA, increased in response to the 3-IM priming series, and sustained for up to 50.5 months in the absence of additional boosters.

Persistence of memory B cells. The onset and duration of PA-specific memory B cell responses were determined for subsets of groups 6 to 12. Memory B cells were first detected at 2 months in the HuAVA group (mean ± standard error [SE], 0.16 ± 0.06 SFU/10^6 cells) and the 1:5 AVA group (0.13 ± 0.05 SFU/10^6 cells). At 5 months, the frequencies increased in the HuAVA (0.32 ± 0.10 SFU/10^6 cells) and 1:5 AVA (0.22 ± 0.08 SFU/10^6 cells) groups. The vaccination at 6 months resulted in levels at 7 months that were significantly different from the control level for the HuAVA (1.06 ± 0.21 SFU/10^6 cells) and 1:5 AVA (0.44 ± 0.12 SFU/10^6 cells) groups. Memory B cell frequencies for the HuAVA and 1:5 AVA groups remained elevated at all time points examined (Fig. 3B).

Frequencies of IFN-γ- and IL-4-secreting cells. The onset and duration of detectable IFN-γ (Th1)- and IL-4 (Th2)-secreting cells were determined as additional measures of AVA-induced Th1/Th2 disposition. Frequencies of IFN-γ- and IL-4-secreting cells above the control levels were detectable at 1 month for all groups except the 1:40 AVA group. The 1:5 AVA group was statistically significantly different from the control group for IFN-γ (mean ± SE, 62.0 ± 19.5 SFU/10^6 cells), and the HuAVA and 1:5 AVA groups were significantly different for IL-4 (23.9 ± 6.2 SFU/10^6 cells and 21.0 ± 6.0 SFU/10^6 cells, respectively). Completing the 3-IM series at 6 months resulted in further increases in IFN-γ- and IL-4-secreting cell frequencies, beginning at 9 months. A vaccine dilution dose response was evident at months 20, 27.25, 33, and 36 for IFN-γ and at months 24 and 26.25 for IL-4. Maximum responses for IL-4 and high levels for IFN-γ occurred at 12.5 months, 6 months after completion of the 3-dose priming series (for HuAVA, mean ± SE of 139.0 ± 25.6 IFN-γ SFU/10^6 cells and 182.9 ± 49.1 IL-4 SFU/10^6 cells; and for 1:5 AVA, 123.2 ± 19.1 IFN-γ SFU/10^6 cells and 188.7 ± 41.9 IL-4 SFU/10^6 cells). In general, the frequencies of IFN-γ- and IL-4-secreting cells remained above control levels from 1 through 50.5 months (Fig. 3C and D).

Duration of protection of 3-IM AVA schedule. Vaccinating with the 3-IM schedule, with dilutions of up to 1:40 AVA, successfully modulated the immune response and provided a gradation of survival frequencies (40 to 100%) for COP analysis (Table 1). Delivered doses of aerosolized *B. anthracis* spores ranged from 37 to 840 LD$_{50}$ equivalents (median, 504; mean, 461; geometric mean, 391) over the 3 challenge time points. There were no statistically significant differences in survival rates between groups of NHPs that received the same vaccine dilution but were challenged at different times (P > 0.05) (not shown) or between vaccinated NHP groups that received the same AVA dilution and were chal-
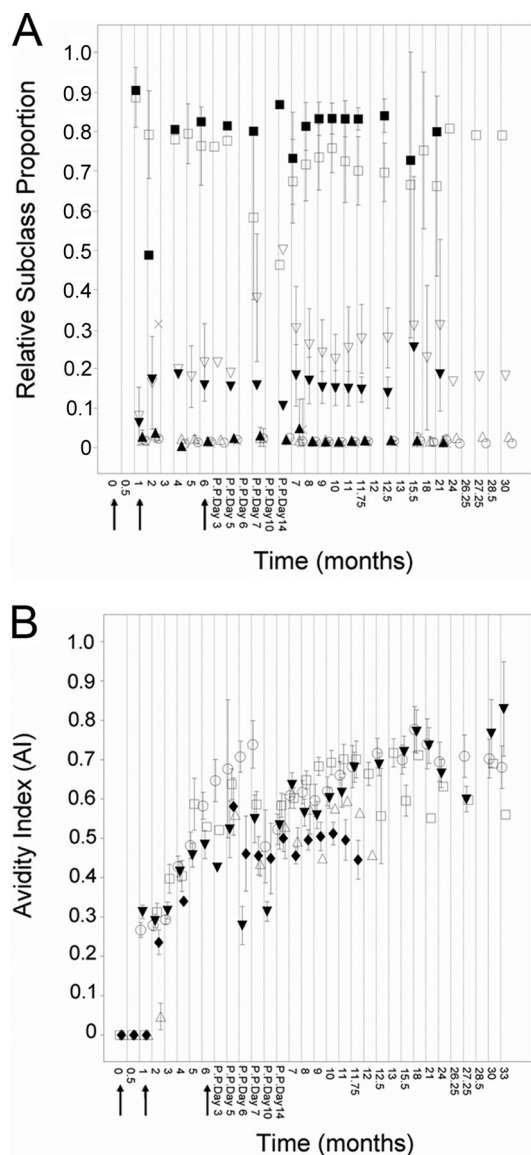
FIG 2 Anti-PA IgG subclass distributions and avidity analyses. (A) Relative subclass levels were determined for samples that had total anti-PA IgG levels of ≥10 µg/ml, had all four subclass IgG ELISAs performed, and had at least one IgG subclass detected. The HuAVA and 1:5 AVA subclass profiles were representative of all of the AVA groups. Error bars represent 95% CI for groups/days with at least 3 samples. Vertical arrows indicate injection time points. PP, days after priming at 6 months. □, HuAVA IgG1; ▽, HuAVA IgG2; △, HuAVA IgG3; ○, HuAVA IgG4; ■, 1:5 AVA IgG1; ▼, 1:5 AVA IgG2; ▲, 1:5 AVA IgG3; ×, 1:5 AVA IgG4. (B) AI determined by dissociation of antibody-antigen complexes in the presence of a chaotropic salt are an indirect measure of the affinity of an antibody for its cognate protein antigen and provide an assessment of immune response maturation. A single vaccination with HuAVA or 1:5 diluted AVA stimulated anti-PA IgG antibodies with measurable AI. Affinity maturation reached its maximum level between 2 and 6 months in response to the 2nd vaccination. The circulating high-avidity antibodies persisted through all time points for which samples met the testing criteria (33 months). Error bars represent 95% CI. ○, HuAVA; ▼, 1:5 AVA; □, 1:10 AVA; ◆, 1:20 AVA; △, 1:40 AVA.

cant difference (Fisher's exact test; $P < 0.05$) between overall survival at 12 months compared to 30 months, there were no statistical differences evident between 12 and 52 months or between 30 and 52 months (Table 4). A series of logistic regression models based on survival was fitted with the explanatory variables of challenge time, infectious challenge dose, and their interaction, with vaccine dilution excepted. These analyses indicated that there were no statistically significant relationships between challenge time or challenge dose and survival. Consequently, the logistic regression models of immunological correlates of protection did not include effects of challenge dose and challenge time.

Logistic regression analysis of immunological correlates of protection. Anti-PA IgG, TNA, and SI at multiple time points were significant predictors of 80% survival probability for all groups at all challenge time points (Tables 5 and 6). The point estimate associated with the 80% probability of survival level in this study varied depending on the time of measurement, following the same pattern as, for example, total anti-PA IgG in Fig. 1. Analogous to month 2 in the AVRP human clinical trial (30), in rhesus macaques an ED_{50} of >338 or an anti-PA IgG response of >30.4 µg/ml at 2 months was predictive of 80% survival probability, independent of the time of challenge. As antibody levels declined prior to completion of the 3-IM priming series, at 3, 4, 5, and 6 months the anti-PA IgG and TNA levels predictive of 80% survival ranged from 3.5 to 8.6 µg/ml and ED_{50}s of 30 to 42, respectively. At PP week 4 (study month 7) following the rapid response to the 6-month vaccination, the estimated 80% predictive levels were 97.3 µg/ml anti-PA IgG and an ED_{50} of 1,243 (Table 5; Fig. 4).

Immune responses PP declined over time. At the time points for aerosol exposure, the anti-PA IgG level and TNA predictive of ≥80% survival were as follows: 7.0 µg/ml and ED_{50} of 92 at 11.75 months, 3.9 µg/ml and ED_{50} of 66 at 30 months, and 3.6 µg/ml and ED_{50} of 34 at 41.75 months (Table 5). Anti-PA IgG and TNA levels after 41.75 months, where detectable, did not have a significant correlation in the logistic regression model.

Because of limited data, fewer time points were associated with survival for the anti-PA IgG subclass levels; however, months 9, 10, 11, and 11.75 were significant for all four subclasses (Table 6 [data for IgG1 are shown]). The predictive value of the IgG subclasses was most likely due to their correlation with total anti-PA IgG (data not shown).

A survival probability of 80% was associated with SI of 4.9 (1 month), 24.1 (2 months), 7.41 (3 months), 6.3 (4 months), 6.9 (5 months), and 68.21 (10 months), although the confidence intervals for the SI at 10 months were large (Table 6). The absence of detectable serum antibody at later time points was not an indication of a loss of protection. For example, there were 31 vaccinated animals with anti-PA IgG levels below the LOD at their respective challenge times, and 21 (64.5%) of them survived subsequent challenge (not shown). The area under the ROC curve was lowest for month 1 (~60%), fluctuated from 70% to 85% throughout the later time points for total anti-PA IgG, anti-PA IgG1, and ED_{50}, and was somewhat lower for SI. The H-L statistic indicated that a lack of fit was uncommon. The TNA data at 2 months and 4 months and the anti-PA IgG data at 4 months did not meet the H-L goodness-of-fit test. No adjustments to the model were made when this lack of fit was noted.

Study month 7 was the only time point for which linear regression analyses indicated a statistically significant intercept, slope,

lenged at the same nominal time interval but different points in the study (e.g., between groups 5 and 8). There was little indication of a trend in survival rates over challenge times within a vaccine dilution (Table 4). Although there was a statistically signifi-
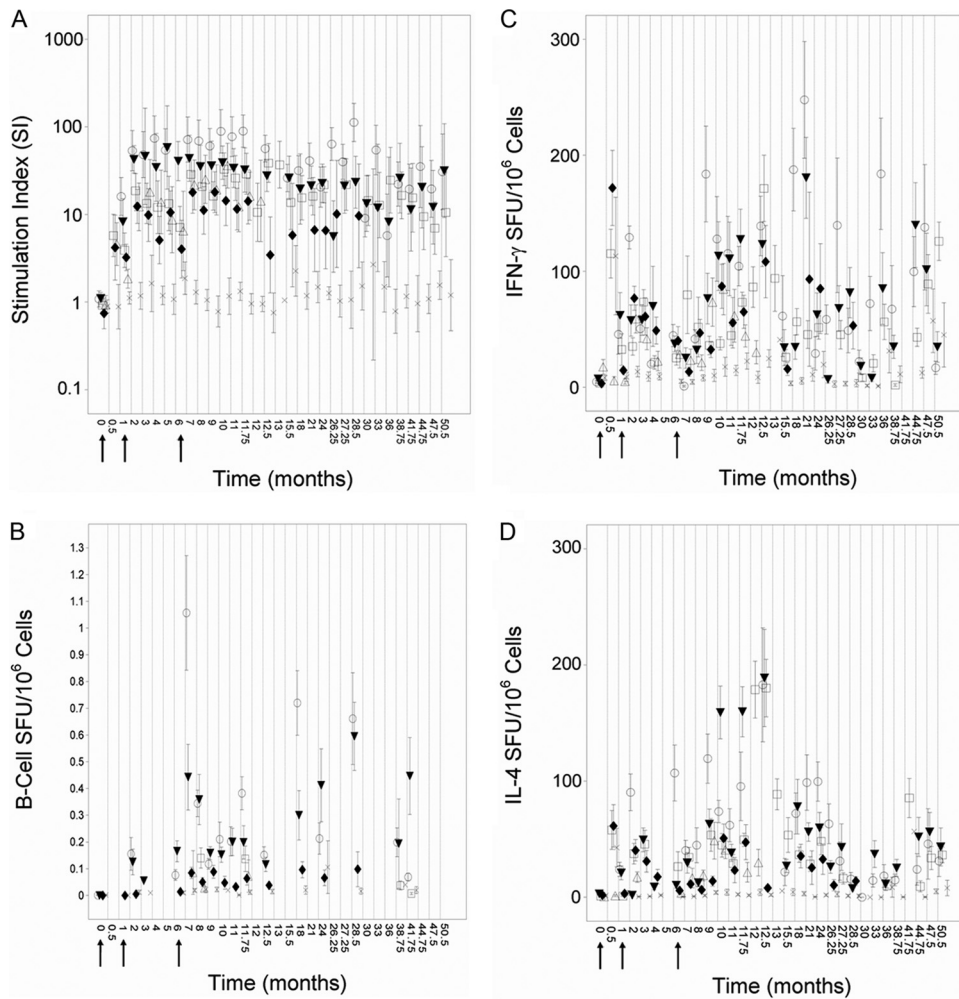
FIG 3 Cellular immune responses to a 3-IM schedule of AVA. (A) T cell SI for each dose group on each study day. Error bars indicate 95% CI. (B) Memory B cells (mean SFU/10^6 IgG-secreting cells) determined by ELISpot analyses. Error bars indicate 1 SE. (C and D) Frequencies of IFN-γ (C)- and IL-4 (D)-secreting cells (mean SFU/10^6 PBMC) determined by ELISpot analyses. Error bars indicate 1 SE. Vertical arrows indicate injection time points. $\bigcirc$, HuAVA; $\blacktriangledown$, 1:5 AVA; $\square$, 1:10 AVA; $\blacklozenge$, 1:20 AVA; $\triangle$, 1:40 AVA; $\times$, controls.

and goodness of fit for both the anti-PA and TNA values. Selection of 7 months as the time point for determining the COP estimate for 80% probability of survival was also supported by ROC analysis, the relative narrowness of the 95% CI for the immune response point estimate, and the *P* value for the slope (Table 5).

Postexposure anti-PA and TNA responses. The onset and magnitude of exposure-induced anti-PA IgG responses in all surviving animals were determined (Fig. 5). PE analyses were independent of the time since last vaccination. Anti-PA IgG and TNA at the day of challenge were statistically significantly higher than

TABLE 4 Comparison of survival rates at different challenge times and AVA dilutions

| Time of challenge (mo) | No. of survivors/no. of challenged animals (%) | | | | | | Fisher's exact test comparison of challenge times over dilutions (*P* value)[a] | |
	HuAVA (undiluted)	1:5 AVA	1:10 AVA	1:20 AVA	1:40 AVA	Combined over dilutions	30 mo	52 mo
12			8/10 (80.0)	11/20 (55.0)	13/20 (65.0)	32/50 (64.0)	0.013	0.217
30	10/10 (100.0)	8/8 (100.0)	6/9 (66.7)	7/8 (87.5)		31/35 (88.6)		0.491
52	8/10 (80.0)	9/9 (100.0)	6/10 (60.0)			23/29 (79.9)		

[a] Fisher's exact test was used to compare the overall survival rates between all pairs of challenge times for the vaccinated rhesus macaques. There was a statistically significant difference between the survival of animals challenged at 12 months and that of animals challenged at 30 months; however, no statistical differences were evident between animals challenged at 12 months and 52 months or between animals challenged at 30 months and 52 months.

TABLE 5 Anti-PA IgG and TNA ED_{50} levels associated with 80% probability of survival in AVA-vaccinated animals at different study time points up to study month 52[a]

Time postvaccination (mo)	Total anti-PA IgG (μg/ml) (95% CI)	Intercept (P value)	Slope (P value)	H-L goodness-of-fit test P value[b]	Area under ROC curve (%)	TNA (ED_{50}) (95% CI)	Intercept (P value)	Slope (P value)	H-L goodness-of-fit test P value[b]	Area under ROC curve (%)
1	6.4 (0, >10max)	0.51 (0.1595)	1.08 (0.0720)	0.715	59.9	41 (20, >10max)	−0.96 (0.3409)	1.46 (0.0466*)	0.715	61.7
2	30.4 (12.5, 170.3)	−0.37 (0.4010)	1.18 (0.0005*)	0.266	73.5	338 (124, 2,443)	−1.26 (0.0677)	1.05 (0.0007*)	0.042	73.0
3	4.8 (1.5, 37.1)	−0.02 (0.9766)	2.06 (0.0240*)	0.930	73.9					
4	4.1 (1.4, 24.4)	0.26 (0.6284)	1.85 (0.0249*)	0.0279	72.7	36 (14, 170)	−1.27 (0.2282)	1.71 (0.0129*)	0.011	70.7
5	3.5 (0.6, 149.2)	0.36 (0.5071)	1.87 (0.0425*)	0.255	69.3	30 (5, 249)	−0.89 (0.4076)	1.54 (0.0301*)	0.759	69.2
6	8.6 (4.7, 1,336.3)	−0.24 (0.6493)	2.62 (0.0161*)	0.114	69.0	42 (25, 205)	−1.94 (0.0633)	2.05 (0.0054*)	0.567	68.7
6.5						5,712 (1,621, >10max)	−3.31 (0.0970)	1.25 (0.0446*)	0.483	70.9
7	97.3 (49.4, 329.7)	−1.72 (0.0256*)	1.56 (0.0003*)	0.241	75.4	1,243 (621, 4,479)	−3.31 (0.0077*)	1.52 (0.0005*)	0.617	74.5
8	25.0 (12.8, 81.1)	−0.84 (0.1002)	1.59 (0.0001*)	0.175	76.2	312 (143, 1,258)	−2.02 (0.0160*)	1.36 (0.0002*)	0.067	75.2
9	14.1 (8.0, 38.0)	−0.83 (0.0797)	1.93 (0.0001*)	0.692	78.0	143 (70, 493)	−1.91 (0.0104*)	1.53 (0.0001*)	0.239	76.9
10	11.6 (6.5, 34.6)	−0.61 (0.1737)	1.87 (0.0002*)	0.440	76.3	124 (64, 422)	−2.00 (0.0090*)	1.62 (0.0001*)	0.187	76.6
11	7.8 (5.0, 18.8)	−0.78 (0.0965)	2.43 (0.0001*)	0.920	77.6	106 (55, 368)	−1.91 (0.0138*)	1.63 (0.0002*)	0.720	75.2
11.75	7.0 (4.6, 16.4)	−0.79 (0.0932)	2.57 (0.0002*)	0.68	77.5	92 (48, 345)	−1.80 (0.0266*)	1.62 (0.0005*)	0.192	72.7
12.5	5.4 (2.5, 15.2)	−1.23 (0.2358)	3.58 (0.0086*)	0.830	85.9	56 (4, 295)	−1.74 (0.2399)	1.79 (0.0209*)	0.705	79.5
15.5	5.6 (2.9, 14.6)	−0.98 (0.2602)	3.18 (0.0060*)	0.710	80.8	60 (23, 207)	−2.63 (0.0696)	2.25 (0.0055*)	0.777	80.7
18	5.0 (2.6, 14.8)	−0.86 (0.3249)	3.22 (0.0101*)	0.797	81.6	49 (23, 149)	−3.15 (0.0423*)	2.68 (0.0042*)	0.959	82.6
21	4.2 (2.5, 11.1)	−1.08 (0.2522)	3.98 (0.0110*)	0.986	81.9	43 (24, 118)	−4.05 (0.0189*)	3.33 (0.0028*)	0.963	86.1
24	3.9 (2.2, 11.1)	−0.87 (0.3467)	3.84 (0.0154*)	0.971	80.6	41 (22, 125)	−3.56 (0.0343*)	3.07 (0.0050*)	0.737	83.4
27.25	3.9 (2.8, 72.3)	−1.96 (0.1323)	5.63 (0.0304*)	0.940	78.2	54 (26, 1,291)	−3.46 (0.0596)	2.80 (0.0189*)	0.676	78.8
30	3.9 (2.9, 35.9)	−2.68 (0.0775)	6.90 (0.0261*)	0.591	83.3	65 (28, 1,644)	−2.99 (0.0817)	2.42 (0.0201*)	0.645	77.2
33	3.9 (0, >10max)	−1.41 (0.3228)	4.70 (0.0870)	0.800	78.6	82 (34, 1,194)	−5.32 (0.0661)	3.50 (0.0263*)	0.957	84.1
36	3.8 (0, >10max)	−1.63 (0.2652)	5.22 (0.0770)	0.825	80.4	61 (19, >10max)	−3.61 (0.1202)	2.80 (0.0415*)	0.402	79.3
38.75	2.9 (0, >10max)	−3.82 (0.2075)	11.40 (0.1234)	0.686	80.8					
41.75	3.6 (0, >10max)	−2.45 (0.1649)	6.84 (0.0599)	0.319	83.3	34 (19, >10max)	−5.31 (0.0804)	4.36 (0.0436*)	0.891	83.3
44.75	2.7 (0, >10max)	−3.36 (0.3642)	10.98 (0.2516)	0.650	75.0					
47.5	2.3 (0, >10max)	−32.09 (0.7874)	89.84 (0.7848)	0.967	80.4					
50.5	2.3 (0, >10max)	−38.75 (0.7651)	108.25 (0.7627)	0.999	80.4					

[a] Assay reportable values are provided for study time points where a statistically significant correlation was observed in logistic regression models. The survival probability represents survival of a challenge at any of the time points after completing the 3-IM priming series. The intercept represents the log odds of animals with a parameter value of 1 surviving challenge. All values are reported on the original scale of measurements. Values greater than 10 times the maximum value are displayed as ">10max." *, statistically significant effect (P < 0.05) of the log-adjusted parameter response on survival.

[b] P values of <0.05 indicate a lack of fit. There were no adjustments for multiple testing.

TABLE 6 Anti-PA IgG1 subclass and SI levels associated with 80% probability of survival at different study time points up to study month 52[a]

Time postvaccination (mo)	Anti-PA IgG1 (μg/ml) (95% CI)	Intercept (P value)	Slope (P value)	Area under ROC curve (%)	H-L goodness-of-fit test P value[b]	SI (95% CI)	Intercept (P value)	Slope (P value)	H-L goodness-of-fit test P value[b]	Area under ROC curve (%)
1						4.9 (2.0, 89.7)	0.51 (0.1428)	1.28 (0.0174*)	0.305	64.8
2						24.1 (12.0, 82.9)	−0.71 (0.1820)	1.52 (0.0004*)	0.295	75.6
3						7.4 (1.5, 36.3)	−0.07 (0.9234)	1.67 (0.0114*)	0.444	77.5
4						6.3 (1.2, 42.4)	0.25 (0.6511)	1.43 (0.0116*)	0.446	76.6
5						6.9 (0.9, 41.8)	0.13 (0.8472)	1.50 (0.0167*)	0.642	77.4
9	1.3 (0.2, 256.2)	1.31 (0.0001*)	0.60 (0.0017*)	75.5	0.753					
10	1.8 (0.2, >10max)	1.27 (<0.0001*)	0.46 (0.0112*)	77.5	0.240	68.2 (15.5, >10max)	0.09 (0.8618)	0.71 (0.0387*)	0.405	59.0
11	0.7 (0.1, 74.1)	1.51 (<0.0001*)	0.66 (0.0013*)	72.6	0.938					
11.75	1.6 (0.2, >10max)	1.28 (0.0007*)	0.53 (0.0131*)	61.0	0.417					
12.5	0.9 (0.0, >10max)	1.41 (0.0138*)	0.72 (0.0453*)	61.1	0.078					
15.5	0.7 (0.0, >10max)	1.52 (0.0095*)	0.77 (0.0391*)	61.1	0.522					

[a] Assay reportable values are provided for study time points where a statistically significant correlation was observed in logistic regression models. The survival probability represents survival of a challenge at any of the time points after completing the 3-IM priming series. The intercept represents the log odds of animals with a parameter value of 1 surviving challenge. All values are reported on the original scale of measurements. Values greater than 10 times the maximum value are displayed as ">10max." *, statistically significant effect ($P < 0.05$) of the log-adjusted parameter response on survival.

[b] P values of <0.05 indicate a lack of fit. There were no adjustments for multiple testing.
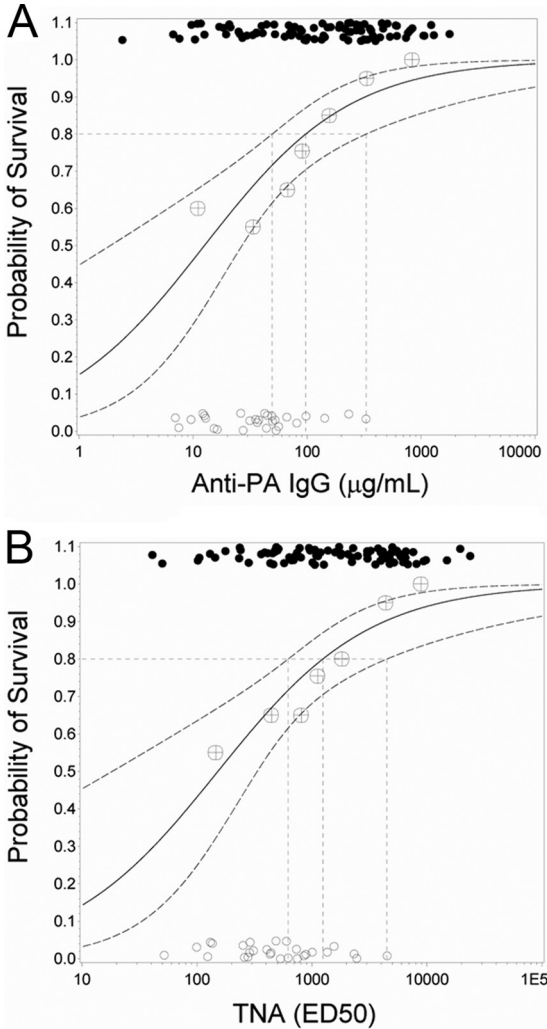

FIG 4 Logistic regression analysis of probability of survival versus humoral immune response. The logistic regression curves (solid lines) illustrate the changes in predicted survival probability with increasing immune responses (dashed lines [95% CI]). The dashed horizontal line indicates the 80% protection level; dashed vertical lines indicate the associated immune response levels. The point estimate associated with an 80% probability of survival in this study varied depending on the time of measurement. Selection of 7 months as the time point for determining the COP estimate for 80% probability of survival is supported by the relative narrowness of the 95% CI and the P value for the slope. Observations were plotted with a slight vertical y axis displacement so that overlapping points may be seen. (A) The anti-PA IgG level associated with an 80% probability of survival at 7 months was 97.3 μg/ml (95% CI = 49.4 to 329.7 μg/ml). (B) The TNA level associated with an 80% probability of survival at 7 months was an ED_{50} of 1,243 (95% CI = 621 to 4,479). ○, animals that died; ●, animals that survived; ⊕, percent surviving animals grouped into bins by 0.5-log_{10} increments of the measured immune response. Bins contained 1 to 34 animals and are for illustration of how the data fit the logistic regression model. Note that in panel A there was only 1 animal in the first bin, and this animal survived inhalation anthrax with a low anti-PA IgG response.

control levels for the HuAVA and 1:5, 1:10, and 1:20 AVA groups. Geometric mean total anti-PA IgG levels ranged from 12.0 μg/ml (HuAVA group; 18/18 animals had a level above the LOD) to 2.2 μg/ml (1:20 AVA group; 16/18 animals had a level above the LOD). Geometric mean TNA results ranged from an ED_{50} of 205 (HuAVA group; 17/17 animals had a level of >0) to an ED_{50} of 12.0 (1:20 AVA group; 17/17 animals had a level of >0). There was
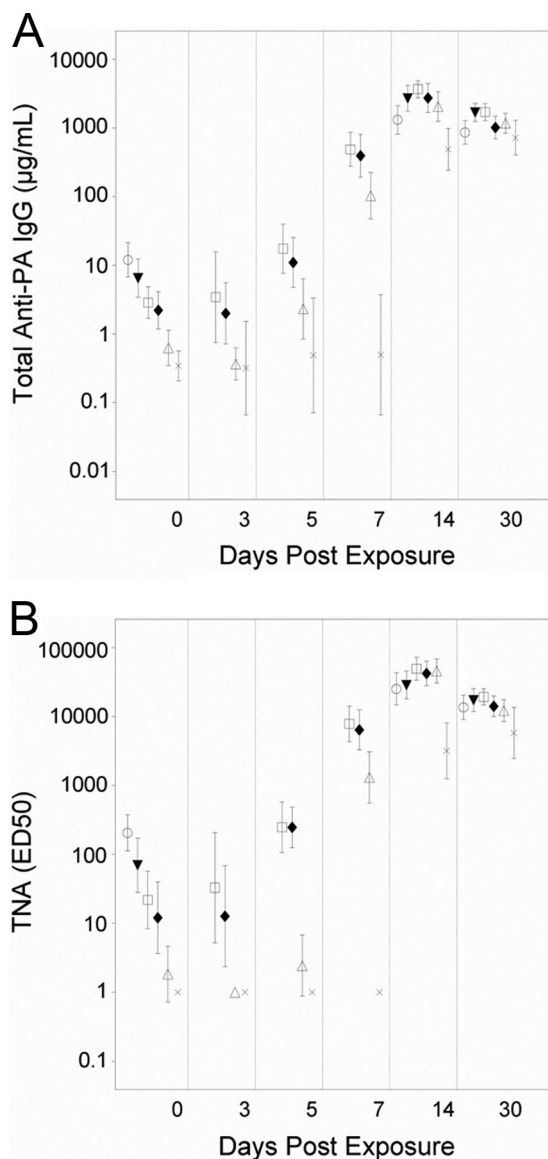
FIG 5 PE serum anti-PA IgG and TNA responses to infection in naïve and AVA-vaccinated rhesus macaques. Rhesus macaques were exposed to target doses of 200 to 400 LD_{50} equivalents of aerosolized *B. anthracis* Ames. The onset and magnitude of infection-induced anti-PA IgG responses in all surviving animals were determined independent of the time since last vaccination. Sample analysis was performed at PE days 3, 5, 7, 14, and 30 for groups challenged at 12 months and at PE days 0, 14, and 30 for groups challenged at 30 and 52 months. Error bars indicate 95% CI. (A) Anti-PA IgG levels (μg/ml). (B) TNA levels (ED_{50}). ○, HuAVA; ▼, 1:5 AVA; □, 1:10 AVA; ◆, 1:20 AVA; △, 1:40 AVA; ✕, controls.

a PE linear vaccine dose response for both anti-PA IgG and TNA. Significant anti-PA IgG and TNA anamnestic responses to infection were detectable by PE day 5 in the 1:10 and 1:20 AVA groups but not in the 1:40 AVA group. The total anti-PA IgG GMC in the 1:10 AVA group increased from 2.9 μg/ml at PE day 0 (20/20 animals had a level above the LOD) to 17.4 μg/ml at PE day 5 (8/8 animals had a level above the LOD). In the 1:20 AVA group, anti-PA IgG levels increased from 2.2 μg/ml at PE day 0 (16/18 animals had a level above the LOD) to 11.0 μg/ml at PE day 5 (11/11 animals had a level above the LOD). TNA increased from

an ED_{50} of 21.8 (20/20 animals had a level of >0) at PE day 0 to an ED_{50} of 246 (7/7 animals had a level of >0) at PE day 5 for the 1:10 AVA group. TNA increased from an ED_{50} of 12.0 (17/17 animals had a level of >0) at day 0 to an ED_{50} of 246 (7/7 animals had a level of >0) at day 5 in the 1:20 AVA group. There was a vaccine dose-response trend for both the anti-PA IgG and TNA ED_{50} on PE days 5 and 7. Specimens at these PE time points were not available for the HuAVA and 1:5 AVA groups.

At PE day 14, all surviving vaccinated animals had significant increases in anti-PA IgG and IgG subclass responses, avidity to PA, and TNA, although a correlation between the original vaccine dilution and the magnitude of the anti-PA response was not evident (Fig. 5). The anti-PA IgG GMC was significantly lower for the HuAVA group (1,314 μg/ml) than the 1:10 AVA group (3,662 μg/ml), demonstrating that the highest vaccine antigen load did not result in the highest PE response. There were no significant differences among the 1:5, 1:10, 1:20, and 1:40 AVA groups. TNA in the vaccine groups ranged from an ED_{50} of 25,111 (HuAVA) to an ED_{50} of 49,367 (1:10 AVA), again confirming that the highest vaccine antigen load did not result in the highest PE responses; however, there were no significant differences in ED_{50} GMT among the vaccinated groups receiving more diluted AVA. In naïve control animals that survived exposure, anti-PA IgG and TNA were not detectable until PE day 7. At PE day 14, the unvaccinated survivors had a significantly lower anti-PA IgG (GMC = 486 μg/ml) and TNA (GMT ED_{50} = 3,167) (Fig. 5).

Vaccinated survivor anti-PA IgG and TNA levels decreased from PE days 14 to 30. In contrast, while lower than responses in the vaccinated animals, the control group antibody levels increased from PE days 14 to 30, indicating that this was a primary response in the vaccine-naïve animals, compared to an anamnestic response in vaccinated animals. At PE day 30, anti-PA IgG GMCs in the vaccinated groups ranged from 861 to 1,699 μg/ml, with maximum responses in the 1:10 AVA group. In controls, the PE day 30 anti-PA IgG GMC was 720 μg/ml, which is not significantly different from those for the HuAVA, 1:20 AVA, and 1:40 AVA groups. The anti-PA IgG GMC was significantly lower for the HuAVA group than for the 1:10 AVA group. There were no significant differences among the 1:5, 1:10, 1:20, and 1:40 AVA groups. TNA in the vaccinated groups were not significantly different (GMT ED_{50} range, 12,176 to 19,303 for 1:40 AVA to 1:10 AVA groups). In control group survivors, the TNA had a GMT ED_{50} of 5,760, which is not significantly different from that for the 1:40 AVA group. Anti-PA IgG avidity PE ranged from 0.53 to 0.82 (AI). In general, AI for HuAVA and 1:5 AVA were significantly higher than 1:20 and 1:40 AVA.

DISCUSSION

These data demonstrate for rhesus macaques that humoral and cellular immune responses determined during and after completion of the 3-IM priming series with HuAVA or diluted AVA were predictive of significant survival against inhalation anthrax at time points distal to vaccination. The 3-IM schedule with no additional booster vaccinations in NHPs was directly analogous to the recently approved 3-dose human priming schedule. To understand more fully the duration of protection afforded by the 3-IM priming series, the NHPs were exposed to high levels of aerosolized *B. anthracis* Ames spores at month 12, 30, or 52, time points consistent with those in the AVRP 4-IM human clinical trial schedule at which an AVA vaccination was replaced by saline injection

(months 12 and 30) or after the final booster vaccination at month 42. Consequently, the infectious challenge at 52 months was a significant test of the 3-IM priming series to provide sustained protection against inhalation anthrax.

Duration of protection of 3-IM AVA schedule in rhesus macaques. In this study, rhesus macaques receiving 3 full or diluted i.m. doses of AVA were exposed to high levels of *B. anthracis* Ames spores at month 12, 30, or 52. The analyses demonstrated that vaccine effectiveness was not affected by spore exposure dose. The sustained levels of survival were significant compared to the survival of unvaccinated controls, and protection did not wane statistically over the study duration ($P \leq 0.05$).

These findings are in contrast to the prevailing perception that AVA immunity wanes rapidly and that annual boosters are required to maintain protection (3, 58) but are in agreement with previous shorter-duration studies in NHPs. The data do, however, differ from studies of alum-based rPA vaccines in rabbits. The NHP and rabbit models of inhalation anthrax have gained increasing prominence for the evaluation of rPA vaccine formulations for humans (15, 34, 52, 54). These animals have been shown to be sensitive to *B. anthracis* infection and to have anthrax pathologies similar to that in humans (10, 54, 59). Data from these models have demonstrated consistently that as few as two doses of AVA and rPA vaccines separated by 7 to 14 days can provide significant short-term protection against acute inhalation anthrax (9, 36, 55). Livingston and coworkers (27) demonstrated that 2 doses of AVA given 8 weeks apart provided 100% protection ($n = 5$) in rhesus macaques up to 1 year after the first vaccination. In contrast, Little and coworkers (22) reported for rabbits that a 2-dose rPA-Alhydrogel formulation (0 and 4 weeks) afforded 74.1% (20/27 animals) and 37.5% (9/24 animals) protection 6 months and 12 months after the first vaccination, respectively. It is unclear whether this rapid loss of protective immunity was a consequence of the vaccine formulation or a feature of the rabbit model. These differences between animal genera emphasize the importance of selecting the most appropriate model for the hypotheses being tested. For evaluations of duration of protective immunity and immune correlates of protection, therefore, the greater immunological similarities of rhesus macaques to humans make these NHPs a more appropriate model (34).

Characteristics of the humoral immune response to AVA. Antigen-stimulated B cells differentiate into antibody-secreting plasmablasts and subsequently into antibody-secreting plasma cells (ASCs) that become the predominant source of antigen-specific antibody production. ASCs have an indeterminate life span, ranging from days to months. A 3-IM schedule of HuAVA and up to 1:20 diluted AVA stimulated dose-dependent, long-term, PA-specific antibody production in NHPs, indicating that this vaccine schedule established long-lived plasma cells. Antibody function was qualitatively similar across all AVA dilution groups.

Maturation of the immune response and Th modulation by AVA was assessed by inspection of the anti-PA IgG subclass distributions. A predominance of IgG2 and IgG3 is considered representative of Th1 responses; IgG1 and IgG4 are considered representative of Th2 responses (33). The limitations of this approach were the dependence on determinations made using cross-reactive anti-human IgG subclass reagents and the fact that rhesus macaques are not reported to produce the equivalent of human IgG3. Nonetheless, cross-reactivity to all 4 subclasses was detected. The presence of both IgG1 and IgG2 was indicative of a

mixed Th1/Th2 response. The predominance of IgG1, however, may be indicative of a Th2-type bias, consistent with previous reports on PA-aluminum hydroxide formulations in rhesus macaques (55).

AI provide an assessment of immune response maturation. A single vaccination with HuAVA or 1:5 diluted AVA stimulated anti-PA IgG antibodies with measurable AI. Affinity maturation reached its maximum level between months 2 and 6 in response to the 2nd vaccination. The circulating high-avidity antibodies persisted through all time points for which samples met the testing criteria (month 33), demonstrating that completion of the 3-IM priming series established populations of long-lived ASCs producing high-affinity anti-PA IgG.

Lethal toxin neutralizing antibodies play a crucial role in protecting animals and humans from aerosol challenge with *B. anthracis* spores. The overall kinetics of the TNA responses and anti-PA IgG levels were highly correlated ($r^2 = 0.92$ and 0.89 at months 2 and 7, respectively) and vaccine dilution dose dependent. The correlation between TNA and anti-PA IgG was similar in each vaccine dilution group, indicating that different antigen loads did not significantly affect the functional activity of the antibody response. Similar to overall anti-PA IgG responses, TNA was of significant duration, demonstrating that the functional activity of anti-PA responses was established early in the 3-IM schedule and sustained for the duration of ASC activity. All 3-IM vaccinated NHPs surviving aerosol challenge with *B. anthracis* Ames spores developed a robust anamnestic response to PA, even if antibody levels prior to challenge were low or undetectable, and all surviving naïve control animals mounted a primary immune response to infection. Surviving animals demonstrated high levels of anti-PA IgG, PA-specific avidity, and TNA. Collectively, these data indicate that while the magnitude of the response to the 3-IM priming series decreases over time, functional high-affinity antitoxin antibodies remain detectable for up to 4 years after the first vaccination. These data clearly indicate that the 3-IM AVA schedule in rhesus macaques, even if diluted, provides robust immunological priming and significant long-term protection against inhalation anthrax.

Characteristics of the cellular immune response to AVA. Establishing immunological memory is critical for the development and maintenance of protective immune responses following vaccination. Immunological memory provides the host with the potential to mount a rapid and protective immune response upon exposure to the pathogen. Whereas antitoxin antibodies react directly to neutralize protein antigens during infection, T cells (CD8 and CD4) recognize cells that present *B. anthracis* antigens on their surfaces. The specific recognition of these antigens by T cells results in either direct killing of the antigen-presenting cells or the release of cytokines. These cytokines provide the necessary signals for the production of antigen-specific antibody.

The development and persistence of circulating PA-specific effector T and B cells were examined by PA-stimulated peripheral blood mononuclear cell proliferation (SI) and determination of the frequencies and duration of IFN-γ- and IL-4-secreting CD4$^+$ cell and memory B cell populations. A significant increase in the SI compared to that of control animals was observed for all groups 1 month after the first vaccination. Completion of the 3-IM priming series at month 6 resulted in the SI remaining significantly higher than that for the control group for all vaccination groups for the study duration, including those groups with a study duration of 52

months. The early-onset and sustained PA-specific lymphocyte function (SI) demonstrated that the schedule provided effective immunological priming of effector T cells.

Animal studies using alternative adjuvants combined with rPA have indicated that specific classes of T lymphocytes representative of a Th1-type response may have an important contribution toward protection (31). Previous studies in humans showed that AVA stimulated the production of $CD4^+$ T cells that recognized multiple epitopes within PA and that these responses were detected in vaccinated and, surprisingly, also in nonvaccinated subjects (18, 19). The frequencies of PA-specific T cells were higher in vaccinees than in nonvaccinees. PA-reactive $CD4^+$ T cells exhibited a Th2 cytokine profile (secreting predominantly IL-5 and IL-13) in vaccinees and a Th0/Th1 cytokine profile (with IFN-γ being the predominant cytokine) in nonvaccinees. Those studies concluded that vaccination with AVA results in commitment of PA-reactive $CD4^+$ T cells to a Th2 lineage and the generation of PA-specific pre-Th2 central memory T cells carrying the $CD45RA^-$ phenotypic marker (19). In this NHP study, development of antigen-specific effector T cell types was examined by measuring the ELISpot frequencies of IFN-γ- and IL-4-secreting cells from PA-stimulated PBMC. IFN-γ- and IL-4-secreting cell frequencies were considered to be representative of Th1 and Th2 responses, respectively. In general, vaccination with AVA resulted in an increase in IFN-γ-secreting cells above the control levels at month 1 for all groups except the 1:40 AVA group, although only the 1:5 AVA group was significantly different from the control group. Completion of the 3-IM priming series at month 6 resulted in a further increase in the frequencies of both IFN-γ- and IL-4-secreting cells by month 9, and the levels remained elevated through month 50.5, primarily in the HuAVA group. The reasons for the delay between completion of the priming series and attainment of maximum responses are unknown. The sustained presence of PA-specific IFN-γ (Th1)- and IL-4 (Th2)-secreting cells, together with the sustained SI, indicated that the 3-IM series provided effective immunological priming and stimulated a mixed Th1/Th2 response in NHPs, in agreement with the observations of Laughlin and coworkers for AVA in humans (19).

$CD4^+$ T cells contribute to activation of naïve B cells to produce clones of germinal center-located, activated B cells and to produce memory B cells and long-lived plasma cells. Circulatory PA-specific memory B cells were detectable in NHPs vaccinated with HuAVA and 1:5 AVA by month 2, were significantly above control levels by month 7, and remained detectable for the study duration, independent of vaccine dilution, at the majority of the time points assayed. In conjunction with the ability to detect anti-PA IgG above control levels for the duration of this 52-month study, these data indicate that initiation and completion of the 3-IM series established long-lived T and B cell memory.

Logistic regression models for analysis of correlates of protection. Prior studies in animal models have shown that anti-PA antibodies contribute to and correlate with protection against inhalation anthrax, although antibody levels may not be complete surrogates of the protective response (50). Passive transfer of anti-PA serum protects mice, guinea pigs, and rabbits from lethal challenge with *B. anthracis* spores (2, 13, 17, 21, 40, 41, 47). Antibodies that bind to PA play a major role in preventing intoxication by LF or edema factor (EF) (47, 60), by blocking the binding of PA to the cell receptor (5, 6, 23, 24, 46, 53), by blocking binding of either LF or EF to PA (47, 60), or as direct inhibitors that cause

structural perturbation of the anthrax toxin complex (38). Depending upon the animal model, anti-PA IgG levels and TNA ED_{50} titers were predictive of survival; for example, anti-PA IgG levels were predictive of survival in rabbits but not guinea pigs or mice, whereas TNA titers were predictive in rabbits (2, 9, 13, 17, 21, 22, 25, 26, 29, 35, 36, 41, 50, 51).

In contrast to previous rabbit studies, however, the data in this NHP study indicate that effector lymphocyte responses (SI) can be a valuable correlate of protection, particularly after vaccination, when circulating anti-PA antibody levels may be low or undetectable (36). As observed for the short duration of protection provided by an rPA vaccine in rabbits (22), it is unclear whether this absence of a correlation with SI in rabbits was a consequence of the 2-dose (0 and 1 month) AVA schedule in that study or a feature of the rabbit model.

In this study, the logistic regression models relating humoral and cellular immune responses to survival established that the practical predictors of ≥80% survival for animals completing the 3-IM series were anti-PA IgG and TNA at month 7 or SI at month 2. The logistic regression analyses demonstrated that the greater the magnitude of response to priming with AVA was, the higher the probability of survival at any time point for up to 3.5 years after completing the schedule. Importantly, these data indicate that although anti-PA IgG, TNA, and SI correlated with protection, there was not a specific protective threshold response above which survival against inhalation anthrax was ensured. At time points distal to vaccination, such as month 42, an anti-PA IgG or TNA response greater than the assay LLOQ (2.3 µg/ml or an ED_{50} of 36, respectively) was still predictive of high levels of survival. It is also important that low or undetectable levels of anti-PA IgG or TNA at the time of infectious challenge were not an indication of waning protection for the 3-IM schedule. Protective immunity did not diminish significantly over time for the HuAVA and 1:5 and 1:10 AVA dose dilution groups.

Conclusions. We concluded that the 3-IM AVA priming series elicited long-term (50.5 months) production of high-affinity PA-specific functional antibody and PA-specific circulating memory B cells and T cells capable of mounting a rapid, elevated, and protective anamnestic response following exposure to high levels of aerosolized *B. anthracis* Ames spores. Humoral and cellular immune responses to 3-IM priming with HuAVA or diluted AVA indicated a mixed Th1/Th2 profile and were predictive of significant survival at time points distal to vaccination when serum anti-PA levels were low or even undetectable. The immunological correlates for ≥80% probability of survival in rhesus macaques completing the 3-IM AVA series were an anti-PA IgG level of >97.3 µg/ml and a TNA ED_{50} titer of >1,243 at month 7, or an SI of >25 at month 2. Correlate anti-PA IgG, TNA, and SI values for ≥80% survival varied with time of measurement, such that there was not a specific protective threshold for the immune response. The 3-IM priming series of undiluted HuAVA and AVA diluted up to 1:10 in rhesus macaques provided significant levels of protection (60 to 100%) against inhalation anthrax for at least 4 years after the first vaccination.

REFERENCES

1. **Abboud N, et al.** 2009. Identification of linear epitopes in *Bacillus anthracis* protective antigen bound by neutralizing antibodies. J. Biol. Chem. **284**:25077–25086.

2. **Beedham RJ, Turnbull PC, Williamson ED.** 2001. Passive transfer of protection against *Bacillus anthracis* infection in a murine model. Vaccine **19**:4409–4416.

3. **Bouzianas DG.** 2010. Current and future medical approaches to combat the anthrax threat. J. Med. Chem. **53**:4305–4331.

4. **Boyer AE, et al.** 2009. Kinetics of lethal factor and poly-D-glutamic acid antigenemia during inhalation anthrax in rhesus macaques. Infect. Immun. **77**:3432–3441.

5. **Chen Z, et al.** 2006. Efficient neutralization of anthrax toxin by chimpanzee monoclonal antibodies against protective antigen. J. Infect. Dis. **193**:625–633.

6. **Cirino NM, et al.** 1999. Disruption of anthrax toxin binding with the use of human antibodies and competitive inhibitors. Infect. Immun. **67**:2957–2963.

7. **Crotty S, Aubert RD, Glidewell J, Ahmed R.** 2004. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. J. Immunol. Methods **286**:111–122.

8. **Crotty S, et al.** 2003. Cutting edge: long-term B cell memory in humans after smallpox vaccination. J. Immunol. **171**:4969–4973.

9. **Fellows PF, et al.** 2001. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by *Bacillus anthracis* isolates of diverse geographical origin. Vaccine **19**:3241–3247.

10. **Fritz DL, et al.** 1995. Pathology of experimental inhalation anthrax in the rhesus monkey. Lab. Invest. **73**:691–702.

11. **Gubbins MJ, et al.** 2006. Production and characterization of neutralizing monoclonal antibodies that recognize an epitope in domain 2 of *Bacillus anthracis* protective antigen. FEMS Immunol. Med. Microbiol. **47**:436–443.

12. **Hanson JF, Taft SC, Weiss AA.** 2006. Neutralizing antibodies and persistence of immunity following anthrax vaccination. Clin. Vaccine Immunol. **13**:208–213.

13. **Hewetson JF, et al.** 2008. An *in vivo* passive protection assay for the evaluation of immunity in AVA-vaccinated individuals. Vaccine **26**:4262–4266.

14. **Hornung RW, Reed L.** 1990. Estimation of average concentration in the presence of nondetectable values. Appl. Occup. Environ. Hyg. **5**:46–51.

15. **Joellenbeck LM, et al.** 2003. An assessment of the CDC Anthrax Vaccine Safety and Efficacy Research Program. Institute of Medicine, National Academies Press, Washington, DC.

16. **Kelly-Cirino CD, Mantis NJ.** 2009. Neutralizing monoclonal antibodies directed against defined linear epitopes on domain 4 of anthrax protective antigen. Infect. Immun. **77**:4859–4867.

17. **Kobiler D, et al.** 2002. Efficiency of protection of guinea pigs against infection with *Bacillus anthracis* spores by passive immunization. Infect. Immun. **70**:544–560.

18. **Kwok WW, et al.** 2008. The anthrax vaccine adsorbed vaccine generates protective antigen (PA)-specific CD4$^+$ T cells with a phenotype distinct from that of naïve PA T cells. Infect. Immun. **76**:4538–4545.

19. **Laughlin EM, et al.** 2007. Antigen-specific CD4$^+$ T cells recognize epitopes of protective antigen following vaccination with an anthrax vaccine. Infect. Immun. **75**:1852–1860.

20. **Li H, et al.** 2008. Standardized, mathematical model-based and validated in vitro analysis of anthrax lethal toxin neutralization. J. Immunol. Methods **333**:89–106.

21. **Little SF, Ivins BE, Fellows PF, Friedlander AM.** 1997. Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea pigs. Infect. Immun. **65**:5171–5175.

22. **Little SF, et al.** 2006. Duration of protection of rabbits after vaccination with *Bacillus anthracis* recombinant protective antigen vaccine. Vaccine **24**:2530–2536.

23. **Little SF, Lowe JR.** 1991. Location of receptor-binding region of protective antigen from *Bacillus anthracis*. Biochem. Biophys. Res. Commun. **180**:531–537.

24. **Little SF, et al.** 1996. Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. Microbiology **142**:707–715.

25. **Little SF, et al.** 2004. Development of an in vitro-based potency assay for anthrax vaccine. Vaccine **22**:2843–2852.

26. **Little SF, Webster WM, Norris SL, Andrews GP.** 2004. Evaluation of an anti-rPA IgG ELISA for measuring the antibody response in mice. Biologicals **32**:62–69.

27. **Livingston BD, Little SF, Luxembourg A, Ellefsen B, Hannaman D.** 2010. Comparative performance of a licensed anthrax vaccine versus electroporation based delivery of a PA encoding DNA vaccine in rhesus macaques. Vaccine **28**:1056–1061.

28. **Longworth E, et al.** 2002. Avidity maturation following vaccination with a meningococcal recombinant hexavalent PorA OMV vaccine in UK infants. Vaccine **20**:2592–2596.

29. **Mabry R, et al.** 2005. Passive protection against anthrax by using a high-affinity antitoxin antibody fragment lacking an Fc region. Infect. Immun. **73**:8362–8368.

30. **Marano N, et al.** 2008. Effects of a reduced dose schedule and intramuscular administration of anthrax vaccine adsorbed on immunogenicity and safety at 7 months: a randomized trial. JAMA **300**:1532–1543.

31. **McBride BW, et al.** 1998. Protective efficacy of a recombinant protective antigen against *Bacillus anthracis* challenge and assessment of immunological markers. Vaccine **16**:810–817.

32. **Pahar B, Li J, Rourke T, Miller CJ, McChesney MB.** 2003. Detection of antigen-specific T cell interferon gamma expression by ELISPOT and cytokine flow cytometry assays in rhesus macaques. J. Immunol. Methods **282**:103–115.

33. **Peterson JD, Herzenberg LA, Vasquez K, Waltenbaugh C.** 1998. Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. Proc. Natl. Acad. Sci. U. S. A. **95**:3071–3076.

34. **Phipps AJ, Premanandan C, Barnewall RE, Lairmore MD.** 2004. Rabbit and nonhuman primate models of toxin-targeting human anthrax vaccines. Microbiol. Mol. Biol. Rev. **68**:617–629.

35. **Pitt ML, et al.** 1999. *In vitro* correlate of immunity in an animal model of inhalational anthrax. J. Appl. Microbiol. **87**:304.

36. **Pitt ML, et al.** 2001. *In vitro* correlate of immunity in a rabbit model of inhalational anthrax. Vaccine **19**:4768–4773.

37. **Pittman PR, Gibbs PH, Cannon TL, Friedlander AM.** 2001. Anthrax vaccine: short-term safety experience in humans. Vaccine **20**:972–978.

38. **Radjainia M, Hyun JK, Leysath CE, Leppla SH, Mitra AK.** 2010. Anthrax toxin-neutralizing antibody reconfigures the protective antigen heptamer into a supercomplex. Proc. Natl. Acad. Sci. U. S. A. **107**:14070–14074.

39. **Reason D, Liberato JJ, Sun Keitel W, Zhou J.** 2009. Frequency and domain specificity of toxin-neutralizing paratopes in the human antibody response to anthrax vaccine adsorbed. Infect. Immun. **77**:2030–2035.

40. **Reuveny S, et al.** 2001. Search for correlates of protective immunity conferred by anthrax vaccine. Infect. Immun. **69**:2888–2893.

41. **Rosenfeld R, et al.** 2009. Isolation and chimerization of a highly neutralizing antibody conferring passive protection against lethal *Bacillus anthracis* infection. PLoS One **4**:e6351. doi:10.1371/journal.pone.0006351.

42. **Sawada-Hirai R, et al.** 2004. Human anti-anthrax protective antigen neutralizing monoclonal antibodies derived from donors vaccinated with anthrax vaccine adsorbed. J. Immune Based Ther. Vaccines **2**:5–20.

43. **Scinicariello F, Engleman CN, Jayashankar L, McClure HM, Attanasio R.** 2004. Rhesus macaque antibody molecules: sequences and heterogeneity of alpha and gamma constant regions. Immunology **111**:66–74.

44. **Semenova VA, et al.** 2004. Mass value assignment of total and subclass

immunoglobulin G in a human standard anthrax reference serum. Clin. Diagn. Lab. Immunol. **11**:919–923.

45. **Shearer MH, Dark RD, Chodosh J, Kennedy RC.** 1999. Comparison and characterization of immunoglobulin G subclasses among primate species. Clin. Diagn. Lab. Immunol. **6**:953–958.

46. **Singh Y, Klimpel KR, Quinn CP, Chaudhary VK, Leppla SH.** 1991. The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. J. Biol. Chem. **266**:15493–15497.

47. **Staats HF, et al.** 2007. *In vitro* and *in vivo* characterization of anthrax anti-protective antigen and anti-lethal factor monoclonal antibodies after passive transfer in a mouse lethal toxin challenge model to define correlates of immunity. Infect. Immun. **75**:5443–5452.

48. **Stratton KR, Durch JS, Lawrence RS (ed).** 2001. Vaccines for the 21st century: a tool for decision making. Institute of Medicine, National Academy Press, Washington, DC.

49. **Taft SC, Weiss AA.** 2008. Neutralizing activity of vaccine-induced antibodies to two *Bacillus anthracis* toxin components, lethal factor and edema factor. Clin. Vaccine Immunol. **15**:71–75.

50. **Turnbull PC, Broster MG, Carman JA, Manchee RJ, Melling J.** 1986. Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Infect. Immun. **52**:356–363.

51. **Turnbull PC, et al.** 2004. Vaccine-induced protection against anthrax in cheetah (*Acinonyx jubatus*) and black rhinoceros (*Diceros bicornis*). Vaccine **22**:3340–3347.

52. **Twenhafel NA, Leffel E, Pitt ML.** 2007. Pathology of inhalational anthrax infection in the African green monkey. Vet. Pathol. **44**:716–721.

53. **Varughese M, Teixeira AV, Liu S, Leppla SH.** 1999. Identification of a receptor-binding region within domain 4 of the protective antigen component of anthrax toxin. Infect. Immun. **67**:1860–1865.

54. **Vasconcelos D, et al.** 2003. Pathology of inhalation anthrax in cynomolgus monkeys (*Macaca fascicularis*). Lab. Invest. **83**:1201–1209.

55. **Welkos S, Friedlander A, Weeks S, Little S, Mendelson I.** 2002. In-vitro characterisation of the phagocytosis and fate of anthrax spores in macrophages and the effects of anti-PA antibody. J. Med. Microbiol. **51**:821–831.

56. **Welkos S, Little S, Friedlander A, Fritz D, Fellows P.** 2001. The role of antibodies to Bacillus anthracis and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. Microbiology **147**:1677–1685.

57. **Williamson ED, et al.** 2005. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. Infect. Immun. **73**:5978–5987.

58. **Wright JG, Quinn CP, Shadomy S, Messonnier N.** 2010. Use of anthrax vaccine in the United States. Recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. MMWR Morb. Mortal. Wkly. Rep. **59**:1–30.

59. **Zaucha GM, Pitt LM, Estep J, Ivins BE, Friedlander AM.** 1998. The pathology of experimental anthrax in rabbits exposed by inhalation and subcutaneous inoculation. Arch. Pathol. Lab. Med. **122**:982–992.

60. **Zhao P, Liang X, Kalbfleisch J, Koo HM, Cao B.** 2003. Neutralizing monoclonal antibody against anthrax lethal factor inhibits intoxication in a mouse model. Hum. Antibodies **12**:129–135.

Supplemental Table A1. Comparison of Human Clinical Trial Vaccination and Booster Schedules with Rhesus Macaque Vaccination and Challenge Schedules.

	Primary Series (months)	Booster Schedule (months)	Total Number of AVA doses	Route	Month 0	Month 0.5	Month 1	Month 6	Month 12	Month 18	Month 30	Month 42	Month 52
Humans	0, 0.5, 1, 6	12, 18, 30, 42	8	SC	AVA	AVA	AVA	AVA	AVA	AVA	AVA	AVA	N/A
	0, 0.5, 1, 6	12, 18, 30, 42	8	IM	AVA	AVA	AVA	AVA	AVA	AVA	AVA	AVA	N/A
	0, 1, 6	12, 18, 30, 42	7	IM	AVA	Saline	AVA	AVA	AVA	AVA	AVA	AVA	N/A
	0, 1, 6	18, 42	5	IM	AVA	Saline	AVA	AVA	Saline	AVA	Saline	AVA	N/A
	0, 1, 6	42	4	IM	AVA	Saline	AVA	AVA	Saline	Saline	Saline	AVA	N/A
Rhesus Macaques	0, 1, 6	None	3	IM	AVA	No Injection	AVA	AVA	Challenge (1:10, 1:20, 1:40 AVA)	No Injection	Challenge (HuAVA, 1:5, 1:10, 1:20 AVA)	No Injection	Challenge (HuAVA, 1:5, 1:10AVA)

In the absence of human efficacy data from the CDC Anthrax Vaccine Research Program (AVRP), the objectives of the rhesus macaque study were to modulate the nonhuman primate (NHP) immune response using a range of AVA dilutions, to evaluate the levels of protection afforded by the dilutions of the AVA from the 3-IM minimal priming series at key time points in the human vaccination booster schedule (12mo and 30mo, the time points in the human schedule where booster vaccinations had been replaced by saline injection), to assess the duration of protection provided by the 3-IM priming by infectious challenge of NHP at 52mo and to establish immunological response profiles to AVA that might facilitate the determination of immune correlates of protection for this vaccine.

The table describes the comparative injection schedules for vaccine (AVA) or placebo (saline), routes of vaccine administration, NHP challenge time points and the NHP AVA dilution groups that were challenged at the 12mo, 30mo and 52mo time points. Survival data are provided in the tables in the main text.